ROLE OF THE WASP VENOM PEPTIDE MASTOPARAN IN THE STUDY OF MECHANISMS INVOLVED IN CELL DEATH

1989

ENG

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
AUG 1989	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Role of the Wasp Venom Peptide Mastoparan in the Study of Mechanisms Involved in Cell Death	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University Of The Health Sciences Bethesda, MD 20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**167**	

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

APPROVAL SHEET

CHOOL OF MEDICINE

TEACHING HOSPITALS
WALTER REED ARMY MEDICAL CENTER
NAVAL HOSPITAL, BETHESDA
MALCOLM GROW AIR FORCE MEDICAL CENTER
WILFORD HALL AIR FORCE MEDICAL CENTER

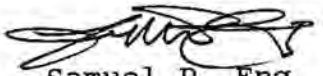
Title of Thesis: **"Role of the Wasp Venom Peptide Mastoparan in the Study of Mechanisms Involved in Cell Death"**

Name of Candidate: Samuel P. Eng
Master of Science
August 23, 1989

Thesis and Abstract Approved:

Committee Chairperson	8 Nov. 1989 Date
Committee Member	8 NOV. 1989 Date
Committee Member	8/11/89 Date
Committee Member	11.9.89 Date
Committee Member	11/13/89 Date

I hereby certify that the use of any copyrighted material in the dissertation entitled: "Role of the wasp venom peptide mastoparan in the study of mechanisms involved in cell death" beyond brief excerpts is with the permission of the copyright owner, and will save and hold harmless the Uniformed Services University of the Health Sciences from any damage which may arise from such copyright violations.



Samuel P. Eng

ABSTRACT

Title of Dissertation: Role of the wasp venom peptide mastoparan in the study of mechanisms involved in cell death.

Samuel P. Eng, Master of Science, 1989

Dissertation directed by: Dr. Chu Shek Lo, Associate Professor, Department of Physiology, USUHS

The effect of mastoparan on membrane function in Madin Darby Canine Kidney (MDCK) cells was studied. Mastoparan increased [^{3}H]-inositol phosphates formation 56% ($p<.001$), 172% ($p<.001$), and 231% ($p<.001$) following treatment of 25, 75, and 100 ug/ml mastoparan, respectively. Mastoparan-induced LDH release reached 8% at 1 min ($p<.005$) and 32% at 15 min ($p<.001$). Mastoparan also mediated the accumulation of inositol-trisphosphate with a concommitant decrease in the level of phosphatidylinositol-bisphosphate. Furthermore, mastoparan significantly activated ($p<.001$) phosphatidylinositol and phosphatidylinositol-phosphate kinase activities in a time and dose dependent fashion. These data suggest that the cellular toxic effects of mastoparan may in part be due to changes in phospholipid metabolism, thereby altering membrane structure and function.

ROLE OF THE WASP VENOM PEPTIDE MASTOPARAN IN THE STUDY OF MECHANISMS INVOLVED IN CELL DEATH

by

Samuel P. Eng

Thesis submitted to the Faculty of the Department of Physiology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Master of Science 1989

DEDICATION

I dedicate this thesis first and foremost to my parents for their trust and support during much more difficult periods.

I also dedicate this thesis to James Eng and Thu-Ha Trinh whose disheartening battle with cancer reaffirmed the nobility of the human spirit.

ACKNOWLEDGEMENTS

I wish to thank Dr. Chu Shek Lo for his patience, guidance, and support throughout my studies. Furthermore, I am most grateful for having been given an opportunity to work in his laboratory.

I would also like to thank Dr. David C. Clough for his assistance, advice, and collaborations.

Table of Contents

Page

LIST OF TABLES

LIST OF FIGURES

Figure | Page

Chapter 1:

INTRODUCTION AND LITERATURE REVIEW

Introduction

Cell death is an important aspect in the pathology of a wide variety of diseases. Possible causes of acute cellular toxicity leading to cell death are insect bites, antibiotics, and toxic chemicals. The mechanisms of cell death may involve one or several processes such as energy deprivation, oxidative damage, membrane dissolution, and inhibition of protein synthesis (Shier, 1985). These pathways in addition to others can be employed by insect venoms to bring about cell damage and eventual cell death.

Bees, wasps, yellow jackets, hornets and ants account for more deaths than any other group of animals (Arnold, 1973; Golden et al., 1989). These insects are all related members of the order Hymenoptera. Members of the order, in particular, bees and ants, are believed to have evolved approximately 100 million years ago from sphecoid wasps, a primitive wasp ancestor (Michener and Brothers, 1974). The criteria for the classification of these species is therefore quite complex and beyond the range of this introduction. However, information on the biology of members of this order can be directed to works performed by Maschwitz and Muhlenbe, (1975).

Among the many insects of the Hymenoptera, wasps are considered to be the most harmful causing serious damage to tissues resulting in severe pain. This applies in particular to social wasps, which build large colonies comprised of 30 to 10,000 or more individuals. This social organization ensures rapid communication

of a disturbance throughout the colony, and stings (primarily used as a defensive weapon) from the colony can be frequent and multiple and are sometimes fatal to an intruder.

Wasp venom when injected intravenously causes a reduction in blood pressure, contraction of smooth muscle preparations, and an edematous reaction on the skin (Nakajima, 1984). A fatal allergic reaction to the venom can evolve rapidly, resulting in upper airway obstruction, severe bronchial dysfunction, cardiac dysfunction, and hypotension (< 60 mm Hg) (Sullivan, 1986).

Biogenic amines in insect venoms

The high mortality rate resulting from stings by wasps, bees and hornets is attributed to specific venom components. The composition, biochemistry, and pharmacology of bee and wasp venoms have been well reviewed by Habermann (1972), Edery et al., (1978), and O'Connor and Peck (1980). These venoms have been shown to contain pharmacologically active amines including acetylcholine, histamine, serotonin, epinephrine, norepinephrine, and dopamine (Schachter and Thain, 1954; Geller et al., 1976; Yoshida et al., 1976).

These so-called biogenic amines are considered common components of vespid and arthropod venom. The amines, though different from those formed in mammals, function quite similarly to a neurotransmitter or autacoid in insect physiology. They are often concentrated in the venom sac of the insect (Ishay, 1978).

Histamine and serotonin were first reported to be the active amines in vespid venom (Jacques and Schachter, 1954 and Schachter, 1970). Of these amines, histamine is the most commonly found in vespid venom and the only one found in substantial quantities in bee venom (Reinert, 1936). In contrast, acetylcholine is found in high concentrations in hornet venom (Bhoola et al., 1961 and Edery et al., 1972). The second most common amines in vespid venom are tyramine and dopamine which are present in concentrations from one fifth to one twentieth of that of histamine (Ishay et al., 1974). Dopamine, also a minor component, has been found in bee venom (Owen, 1971 and Banks et al., 1976).

Catecholamines, such as norepinephrine (recognized as a neurotransmitter in insects) and epinephrine, are only present as minor constituents in bee and wasp venom (Edery et al., 1972; Nakajima et al., 1983; Ishay et al., 1974; Owen, 1971; Geller et al., 1976; Owen and Bridges, 1982). Though the quantities of catecholamines reported are variable, the amounts reported are not sufficient to produce any pronounced pharmacological effects in mammmals, but may be quite effective in some insects. The variability in the quantities observed can be due to the relative instability and rapid oxidation of the catechol group (Edery et al., 1978). Conversly, the aminergic components may be age-related and the variability may be due to samplings from insects of different age groups (Owen and Bridges, 1982).

Most of these amines are derived from amino acids which are components of the venom. The presence of the aminergic pre-

cursors would infer the activation of the corresponding enzymes in the venom gland. The relative activity of these enzymes will determine the proportionate concentrations of biogenic amines present in the venom. Nevertheless, whichever biogenic amine is formed, it will make a substantial contribution to the lethality of the venom through synergistic action with other components of the venom.

Active peptides in insect venoms

These insect venoms also contain enzymes such as hyaluronidase, phospholipase, and active peptides such as kinins in both wasp and hornet venom but not in bee venom (Schachter and Thain, 1954; Geller et al., 1976; Yoshida et al., 1976; Habermann and Neumann, 1957, 1972; Shipolini, 1971).

Kinins were first reported to be active peptides, found in wasp venom, that cause contraction of isolated smooth muscle preparations (Schather and Thain, 1954). This "wasp kinin" was found to exhibit pharmacological behavior similar to bradykinin (Rocha e Silva et al., 1949). In addition, a similar "hornet kinin" was found in the venom of hornets (Bhoola et al., 1961). Currently, 11 types of wasp kinins have been sequenced, all of which exhibit a bradykinin sequence in the molecule (Kishimura et al., 1976; Yasuhara et al., 1977; Yasuhara et al., 1983; Yoshida et al., 1976; Undenfriend et al., 1967; Watanabe et al., 1976; Ueno et al., 1977). Hornet kinins also exhibit a bradykinin sequence,

but unlike wasp kinins, proline at position 3 is converted to hydroxy-proline.

Enzymes such as hyaluronidase are present in both bees and wasps (Edery et al., 1972), and whether there are differences at the molecular level remains to be seen. Furthermore, phospholipases, present in both bee and wasp venom, appear to differ from each other on an immunological basis (King et al., 1978).

Although insect venoms have many components that may act synergistically with other components to cause cell death and produce an inflammatory response, it is the venom polypeptides which are principally responsible for causing anaphalactic shock and eventual death in hypersensitive individuals (Barr, 1974).

The major allergens in honey bees (Apis mellifera) that have been identified are phospholipase A_2, hyaluronidase, acid phosphatase, and melittin. Melittin, NH_3-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-$CONH_2$, is a 26 residue polypeptide found exclusively in bee venom and consists of an N-terminal hydrophobic region and a C-terminal polar sequence. This unique structure is responsible for the peptide's dramatic effects on the membrane. The peptide is capable of spontaneously assimilating into the lipid bilayer, often resulting in profound consequences (Knoppel et al., 1979). It is a potent stimulator of phospholipase A_2 activity (Mollay et al., 1976; Yunes et al., 1977; Shier, 1979), and a powerful cytolytic agent (Terwillinger and Eisenberg, 1982a and 1982b; Habermann, 1972; Sessa et al., 1969). In

addition, melittin is able to cause changes in mitochondrial respiration (Olson et al., 1974), and alters adenylate cyclase activity (Lad and Shier, 1980).

Although the components of wasp, hornet, and yellow jacket venom (Tu, 1977; Habermann, 1972; Edery et al., 1978) have not been as well studied as the honey bee venom, the major allergens identified are protein antigen 5, phospholipases, and hyaluronidase (King et al., 1978, 1983). Though it has no known enzymatic or biological activity to date, antigen 5 is the protein with the greatest allergic activity (histamine release) on a per weight basis (King et al., 1978, 1983). The vespid venom, unlike the honey bee (Apis mellifera) venom, has phospholipase B in addition to phospholipase A_2, proteases, and the peptide mastoparan (Nakajima, 1984 and Hirai et al., 1979a and 1979b). Mastoparan, Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-LeuNH_2, is a tetradecapeptide present in the venom of the wasp Vespula lewisii and has a potent effect in causing rat peritoneal mast cell degranulation and histamine release (Hirai et al., 1979b, 1980). Though Jacques and Schachter reported in 1954 a histamine-releasing factor present in wasp venom, it was not unitl 1979 that Hirai et al. isolated, characterized, and chemically synthesized mastoparan. Subsequently, similar tetradecapeptides of different amino acid sequences were found in the venoms of Vespa xanthopera (Hirai et al., 1979), Vespa mandarina (Hirai et al., 1981), Vespa orientalis (Nazimov et al., 1980), and Vespa tropica (Yasuhara et al., 1983).

Mastoparan has been shown to elicit many effects. At a minimum concentration of 5 ug/ml, mastoparan from Vespa lewisii caused the degranulation of rat peritoneal mast cells and histamine release (Hirai et al., 1979b). Mastoparans from wasps other than Vespa lewisii, for example, mastoparan-X from Vespa xanthopera, did not show such effects at the same concentration (Hirai et al., 1979a).

Mastoparan can enhance ion permeability in membranes of mast cells through modification of membrane structure and function (Okumura et al., 1981). The peptide acts on adrenal chromaffin cells to release catecholamines (Kuroda et al., 1980). Furthermore, it has been demonstrated that mastoparan induces arachidonic acid release from egg liposomes and rat peritoneal mast cells suggesting that this agent activates phospholipase activity (Argiolas and Pisano, 1983). The arachidonic acid that is subsequently released from the membrane phospholipid by phospholipase A_2 becomes an important source from which the mediators of inflammation, such as prostaglandins, are derived. Furthermore, the release of arachidonic acid has been implicated in hormonal responses (Schrey and Rubin, 1979). For example, it has been shown that gonadotropin-releasing hormone (GnRH) increases arachidonic acid release from rat anterior pituitary cells (Noar and Catt, 1981). Moreover, the arachidonic acid may mediate, in part, the intracellular regulation of hormone secretion (Camoratto and Grandison, 1985; Kolesnick et al., 1984). What additional effects mastoparan has on other phospholipases remains to be

determined. It is suggested, however, that mastoparan is involved in the increase in phospholipid turnover which results in the hydrolysis of the substrate phosphatidylinositol 4,5 bis-phosphate and the subsequent generation of two second messengers, diacylglycerol and inositol trisphosphate (Okano et al., 1985). Similar results are also obtained when various agents, including hormones and neurotransmitters, interact with cell surface receptors subsequently activating phospholipase C which would result in the analogous production of the same two messengers (Michell, 1975; Slivka and Insel, 1987). The mechanism by which mastoparan increases phospholipid metabolism remains to be elucidated since it does not appear to be "receptor" mediated (Higashijima, et al., 1988).

Mastoparan is believed to interact directly with the phospholipids (Higashijima et al., 1983; Berridge et al., 1983). Since membrane phospholipids are known to regulate membrane permeability, hydrophobic binding of mastoparan to membrane phospholipids may initiate the pathogenesis of cell injury by altering membrane structure and function. Consequently, studies involving a synthesized peptide such as mastoparan would give information on the role of phospholipid metabolism and membrane function in cell injury and death. The wasp venom peptide mastoparan lacks the severe lytic effects of melittin. Furthermore, mastoparan interacts with the phospholipid membrane through hydrophobic interactions rather than electrostatic interactions as with melittin (Miyazawa and Hagashij, 1984). These

studies provide insight into mechanisms related to cell death. In addition, mastoparan appears to be a valuable tool for studying the pathophysiological processes venom peptides have in man.

CHAPTER 2:

MATERIALS AND METHODS

Materials

MDCK cells were obtained from the American Type Culture Collection (Bethesda, MD). Mastoparan, fatty acid free bovine serum albumin, Tris-ATP, phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2), alpha-aminoisobutyric acid, p-enolpyruvate, pyruvate kinase, ouabain and the LDH assay kit #226-UV were purchased from Sigma Chemicals (St. Louis, MO). Dulbecco's Phosphate Buffered Saline, Dulbecco's modified Eagle's medium (DME medium), 2 mM glutamine, and penicillin-streptomycin were from Gibco (Grand Island, N.Y.). Bovine calf serum was obtained from Hyclone (Logan, UT). myo-[^{3}H]inositol, [gamma-^{32}P] adenosine triphosphate, and [^{3}H]alpha-aminoisobutyric acid were purchased from New England Nuclear. Tissue culture plates and materials were obtained from Costar (Cambridge, MA). All other chemicals were of reagent grade.

Cell Culture Methods

MDCK cells ($4X10^4$) were plated onto each well of the 24 well plate. The cells were grown in DME medium supplemented with 10% fetal bovine calf serum, 2mM glutamine, 50 units penicillin G per 500 ml and 50 ug streptomycin sulfate per ml at 37°C in a humidified atmosphere of 95% air/5% CO_2. The medium was changed twice per week. After the fifth day the wells were confluent and contained $7.5 \pm 1.5 \times 10^5$ cells (comparable to $1.5 \pm .05$ mg of protein/well and 50 ± 5 ug DNA/well; n=8). Experiments were performed on fifth, sixth, or seventh day cultures without any

significant changes in cell number.

Quantitation of [^{3}H]-inositol phospholipid hydrolysis

Methods used for the analysis of cellular phospholipid hydrolysis were described by Berridge et al., (1982) and modified by Beavan et al., (1984) and Maeyama et al., (1986). At least 96 hours after plating, the MDCK cultures were labelled for 12 hours with myo-[2-^{3}H]inositol (1.0 Ci/well; New England Nuclear). An aliquot was removed and the amount of radioisotope that had been incorporated into the cells' lipids showed a dpm range of 30,000-60,000. The labelled cells were washed three times with 1 ml of Dulbecco's Phosphate Buffered Saline (Gibco). These cells were incubated at 37°C for 10 minutes with Dulbecco's Phosphate Buffered Saline supplemented with 0.1% Bovine Serum Albumin (Sigma, St. Loius, MO) and 10mM LiCl. Mastoparan (15 ug) was added to the cultures in a 10 ul volume of double distilled water and was allowed to incubate for specified times. At the end of incubation an aliquot was removed and analyzed for LDH activity and the incubation was terminated by the addition of chloroform/methanol/4N HCl (100/200/2, by volume). This mixture was allowed to separate into an aqueous and organic phase. The aqueous phase containing inositol and organic phosphates was separated on 1 ml formate form anion exchange column (Downes and Michell, 1981) by elution with formate buffers of increasing ionic strength as described by Berridge et al. (1983) and modified by our laboratory. Total [^{3}H]inositol phosphates were eluted with 1.6 M ammonium formate in

100 mM formic acid. To analyze individual inositol phosphates, inositol mono-phosphate (IP_1), inositol bisphosphate (IP_2), and inositol tris-phosphate (IP_3) were eluted with 50 mM ammonium formate in 100 mM formic acid, 400 mM ammonium formate in 100 mM formic acid, and 720 mM ammonium formate in 100 mM formic acid, respectively. Total inositol phospholipids were quantitated by counting the radioactivity of the organic phase. The individual inositol phospholipids in the organic phase were seperated on oxalate impregnated silica gel using the solvent system of Jolles, et al., (1981). Prior to use, the silica gel plates were soaked in methanol containing 0.6% potassium oxalate and 1 mM EDTA for 30 seconds, dried and activated at 110°C for 30 minutes. Organic phase samples were mixed with 5 ug each of the phosphatidyl-inositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2) standards, and evaporated to dryness under a stream of N_2. These samples containing the dried lipid residues were re-dissolved in chloroform and applied to the silica gel plates. In addition, the unlabeled standards were applied to an adjacent lane on the same plate. The plates were developed in a Whatman paper-lined chamber saturated with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by volume) for 90 minutes. After development, the areas containing the lipids were identified by exposing the plates to iodine vapor. Upon sublimation of the iodine stain, the identifiable areas of the silica gel plates were scraped into scintillation vials and the lipid residues resuspended in scintillation fluid (Beckman Ready

Safe). The radioactivity was quantitated on a liquid scintillation counter (Searle, Mark III).

Quantitation of lactate dehydrogenase activity

The aliquot which was obtained from the supernatant of MDCK cells treated with the toxin was analyzed for lactate dehydrogenase (LDH) activity using the Sigma #226-UV assay kit. The LDH activity was expressed as units per litre (one international unit of enzyme will hydrolyze one micromole of substrate per minute) as well as the percentage of total LDH released. Total LDH was determined from aliquots taken from culture wells of MDCK cells treated with 0.1% Triton X-100 in phosphate buffered saline at 37°C for 10 minutes.

Preparation of purified plasma membranes

Purified plasma membranes were prepared from cultured MDCK cells. All preparations were carried out at 4°C. Cells were collected from two 125 cm^2 flasks and centrifuged at low speed (100 x g) for five minutes and the cell pellet resuspended in a buffer consisting of 50 mM Tris-HCl, 20 mM $MgCl_2$, and 1 mM EGTA. This resuspension was homogenized with a Teflon-glass Potter-Elvehjam homogenizer (20 strokes) and loaded onto a discontinuous sucrose gradient composed of the following components: 10% sucrose 3-5 ml, 20% sucrose 3-5 ml, 30% sucrose 3-5 ml, 40% sucrose 3-5 ml. These gradients were centrifuged for 1.5 hours at 100,000 x g in a Sorvall OTD-50 ultracentrifuge with a swinging bucket

rotor AH-627. The fraction found at the top of the 40% sucrose was collected and diluted in kinase buffer (50 mM Tris-HCl (pH 7.0), 20 mM $MgCl_2$, 1 mM EGTA) and stored at -20°C as the final purified plasma membrane preparation. Aliquots of this preparation were taken for protein analysis.

Identification of phosphatidylinositol (PI) kinase activity in the plasma membrane of the MDCK cells

Plasma membrane from the cell homogenate was prepared by discontinuous sucrose gradient centrifugation. Four fractions were obtained: Fraction A corresponded to the uppermost cytosolic fraction. Fraction B, C, and D corresponded to the fractions collected at the interface between 10-20%, 20-30%, and 30-40% sucrose, respectively. Although the protein content of fraction D constituted only 18% of the total protein, it contained 69% of the total Na^+,K^+-ATPase activity and 55% of the total PI kinase activity. These findings suggest that fraction D is plasma membrane enriched. An increase in the amount of protein in fraction D resulted in a proportionate increase in the PIP level (7.4 ± 1.2, 11.8 ± 1.6, and 22 ± 2 pmols of PIP per 10 min per 19 ug , 39 ug, and 78 ug of membrane protein, respectively). Thus, fraction "D" was used in all subsequent experiments.

Identification of phosphatidylinositol 4-phosphate (PIP)

Since the plasma membrane contains other phosphate acceptors, it is important to determine if the membrane component being

phosphorylated under our experimental conditions is phosphatidyl-inositol (PI). Since neomycin binds with high affinity to PIP (Lodhi et al., 1979 and Schacht, 1978), an immobilized neomycin column was prepared to separate PIP from other phospholipids. Neomycin was coupled to the glycophosphate CPG (200/400 mesh with 200 A pore diameter, Pierce Co., Rockford, IL) supports according to Schacht, (1978). In three separate experiments, fraction "D" isolated from MDCK cells previously labelled with [^{3}H] inositol overnight (14-18 h) was incubated with [gamma-^{32}P]ATP for 10 min at 30°C and then passed through the neomycin column. The inositol phospholipids eluted by 150 mM and 600 mM ammonium acetate were found to co-migrate with the PIP and PIP_2 standards, respectively, in the thin layer chromatography plate. [^{3}H] (4800 ± 200 dpm) and [^{32}P] (450 ± 25 dpm) radioactivities were detected in the PIP molecules indicating phosphorylation of the [^{3}H]inositol-labeled PI molecule. In swine erythrocyte ghost, it was found that no membrane lipids other than the inositol phospholipids were labeled by [gamma-^{32}P] ATP under similar assay conditions (Buckley and Hawthorne, 1972).

Assay of phosphatidylinositol (PI) and phosphatidylinositol phosphate (PIP) kinases in purified plasma membranes of cultured MDCK cells

PI kinase and PIP kinase were assayed using techniques described by Cockcroft et al. (1985) and Michell et al. (1967). The reaction mixture consisted of 50 mM Tris-HCl (pH 7.0), 20 mM

$MgCl_2$, 1 mM EGTA, and 5 mM [gamma-^{32}P]ATP (specific activity: 10 uCi/mmol), and purified plasma membranes from cultured MDCK cells (a maximum of 15 ug protein). The total volume of the mixture was 150 ul. The mixture was incubated at 30°C for a specified time with a specified amount of toxin and the reaction was terminated by the addition of chloroform/methanol/4 N HCl (10/20/.2). Using methods previously described, the lipids were extracted and concentrated under a small stream of N_2 and applied to oxalate impregnated silica gel for seperation (Jolles et al., 1981). PI kinase and PIP kinase activity were measured as the amount [^{32}P]incorporated into the di- and tri-phosphoinositides. The amount of PIP and PIP_2 synthesized was expressed as dpm per mg protein, assuming that only the terminal phosphate group is incorporated into the phospholipid. As pointed out by Cockcroft et al. (1985), the kinase activity may be underestimated because the method has ignored the possible presence of the phosphatases in the membrane preparations.

Preparation of isolated cell suspension from cultured MDCK cells

Isolated cell suspensions of MDCK cells were prepared from 75cm^2 flasks which were seeded with 7 X 10^6 cells. Four days after seeding, these confluent cultures were rinsed 2X with 10 ml Dulbecco's Phosphate Buffered Saline (PBS; Gibco, Grand Island, N.Y.) and aspirated. Upon aspiration, 10 ml of Trypsin Solution (NaCl 8g/l; KCl 0.4g/l; Dextrose 1g/L; $NaHCO_3$ 0.58g/L;

trypsin 0.5g/L; EDTA 0.2 g/L) were added and the flask was gently shaken for 1 minute and decanted. These cultures were then incubated at 37°C for 30 minutes in an atmosphere of 95% air/5% CO_2. Upon completion, the cells were resuspended in 10 ml PBS and transferred to centrifuge tubes and centrifuged for 4-5 minutes at 250 rpm (500 X g) in a IEC centrifuge (Damon). The supernatant was removed and the pellet was resuspended with 10 ml PBS and centrifuged under the previous conditions for two cycles. After the second cycle of centrifugation and resuspension, the pellet was resuspended in 5 ml PBS making a final cell concentration of 3.5-4.0 X 10^6 cells/ml (1.5 mg protein/ml). Viability was tested by incubating the cells with ethidium bromide (a final concentration of 1 ug/ml) and fluorescein diacetate (a final concentration of 16 ug/ml) in PBS for 3 min. at room temperature. At the end of 3 min., a drop of cell suspension was observed under a Zeiss microscope equipped with an IV F1 epifluorescent condenser. Intact cells yield a bright green fluorescent complex as a result of the accumulated products of fluorescein diacetate hydrolysis (Rotman and Paperwasten, 1966). Ethidium bromide penetrates intact cells slowly but enters damaged cells rapidly (Edidin, 1970). It yields a bright red color complex with the DNA. On the basis of this differential technique, viable cells can be distinquished from dead cells.

Determination of alpha-aminoisobutyric acid (AIB) uptake in isolated cell suspensions of MDCK cells

Isolated cell suspensions used for AIB uptake studies were prepared from these cultures using procedures previously described. In each study, 170 ul (comparable to 250-350ug protein) of the isolated cell suspension was combined either with 20 ul of mastoparan or 20ul of Phosphate Buffered Saline (Gibco) and placed in 12mm X 75mm plastic tubes and incubated in a 37°C water bath. This incubation mixture was then pulsed with 10 ul ^{3}H-AIB solution (50 ul ^{3}H-AIB at 1 uCi/ul + 450 ul PBS) giving 1.0 uCi/sample for various periods of time according to the individual experimental protocol. Upon completion of the incubation, 200 ul of the mixture was placed on 25mm membrane filters with 0.45 um pores (Gelman Science, Ann Arbor, MI). These membrane filters were washed 5X with 5 ml of 1mM cold AIB in ice cold 0.9% NaCl using a vacuum manifold (Amicon). The filters were transferred to counting vials and counted in Aquasol-II (NEN/Dupont) with an Analytic II Scintillation Counter (Searle).

Determiantion of AIB efflux

Isolated cell suspensions (1.5mg - 3.5mg protein) were incubated in PBS containing ^{3}H-AIB (10 uCi/ml) for 30 minutes at 37°C. At the end of the incubation, the loaded cells were rinsed three times in PBS containing 5 mM non-labelled AIB. The cell pellet was resuspended in an "efflux" medium containing 5 mM non-labelled AIB and 1 mM ouabain in PBS. Aliquots (200 ul) of the

mixture were collected at different times and passed through a glass wool column (1 cm thick). AIB efflux was quantitated by measuring the radioactivity of a known amount of eluate in Aquasol-II with an Analytic II liquid scintillation counter, and the data expressed as nmoles ^{3}H-AIB effluxed from the cells per mg protein.

Preparation of Na^+,K^+-ATPase

Male, Sprague-Dawley rats (120-200g body weight) were maintained on rat chow (Agway 1257) ad libitum. Rats were decapitated, bled, and both kidneys removed. The kidneys were decapsulated, the papillae dissected out and the remainder of the kidneys (including the cortex and medulla) were pooled and weighed. Plasma membrane enriched fractions were obtained using a modification of Jorgensen's method (1974). All procedures were carried out at 0-4°C.

One gram wet weight of pooled renal tissue was suspended in 10 ml of homogenizing solution containing 5 mM EDTA, 30 mM histidine, 250 mM sucrose, pH 7.4, and homogenized in a teflon-glass, motor driven homogenizer (10 strokes). The crude homogenate was centrifuged at 1,000 x g for 10 min in a Sorvall SS-34 rotor. The supernatant was carefully removed with a Pasteur pipette and the resulting pellet resuspended with half the original volume of homogenization solution and processed through two additional cycles of homogenization and centrifugation. The final 1,000 x g pellet was discarded and the pooled supernatants centrifuged in the same rotor at 10,000 x g for 10 minutes. The supernatant was carefully

removed from the bilayered pellet and the upper loose layer (L fraction) which contained mostly plasma membrane vesicles (Lo et al., 1976) collected with a Pasteur pipette and resuspended in 0.5 ml of homogenization solution. The L fraction was aliquoted into several Eppendorf tubes and stored at -20°C until analyzed for Na^+,K^+-ATPase activity.

Determination of Na^+,K^+-ATPase activity

Male Sprague-Dawley rats were decapitated and the kidneys removed as described previously (Jorgenson, 1974). Approximately 15 ug of L fraction protein was incubated in 1.0 ml of a medium which contained 1 mM EDTA, 20 mM KCl, 3 mM $MgCl_2$, 100 mM NaCl, 3.0 mM Tris-ATP, and 100 mM Tris-HCl (pH 7.4 at 37°C), or in a medium of the same composition but in the presence of 1 mM ouabain. The mixture was pre-incubated for 10 minutes at 37°C in the absence of ATP and the reaction started by the addition of Tris-ATP. The reaction was terminated after 15 minutes by the addition of 0.2 ml of 30% ice-cold trichloroacetic acid. Inorganic phosphate (Pi) was determined by the Fiske and Subbarow method (1925). The activity was expressed as the difference between Pi released in the presence and absence of 1 mM Ouabain. Specific activity was expressed as umoles ATP hydrolyzed per mg protein per hour.

Determination of K_D for ATP, sodium, and potassium

The effect of mastoparan on the K_D's (apparent dissociation constant) of the rat kidney Na^+,K^+-ATPase for ATP, Na^+, and K^+ were

determined in an ATP regenerating system consisting of p-enolpyruvate and pyruvate kinase (Schwartz et al., 1969). The pyruvate kinase was dialyzed against 1 l of distillled water for 12 hr. at 0°-4°C prior to use. To determine the K_D's for Na^+, K^+, and ATP the concentration of each of the three components was varied individually in separate experiments and the concentration of the remaining two components was held constant at the concentrations given below. Approximately 15 ug of the L fraction was added to 800 ul of a medium (pre-incubated at 37°C) which contained 100 mM NaCl, 20 mM KCl, 1 mM EDTA, 3 mM $MgCl_2$, and 100 mM Tris, (pH 7.4). Tandem tubes contained 1 mM ouabain. The incubation was started by the addition of Tris-ATP to give a final concentration of 3 mM or a range of concentrations to determine the K_D for ATP. The reaction was terminated after 15 minutes by the addition of 0.2 ml of ice cold 30% trichloroacetic acid.

Determination of phosphorylated intermediate

All assays were carried out at 0°-4°C. The phosphorylated intermediate was generated in 400 ul of a reaction mixture that contained 100 mM NaCl, 100 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, 1.0 uM [gamma-^{32}P]ATP (1.87 mCi/ml), pH 7.4, and 25-40 ug of L fraction in Eppendorf tubes. Tandem tubes containing 20 mM KCl instead of 100 mM NaCl were used for the measurement of non-specific phosphorylation. The reaction was initiated by the addition of [gamma-^{32}P]ATP and was terminated after 15 seconds by the addition of 0.1 ml of 30% trichloroacetic acid. The mixture was centrifuged

in an Eppendorf centrifuge (model 5412) at high speed for 1 minute. The pellet was washed twice with 3 mM ATP in 100 mM Tris-HCl,(pH 6.8). After the final wash, the Eppendorf tubes were inverted and dried. The pellet was dissolved in 200 ul of 1N NaOH for 1 hr. The samples were removed and titrated with 1 N HCl and placed in scintillation vials containing 10 ml of Aquasol for quantitation in a Mark-II spectrophotometer (Searle).

Modulation of mastoparan induced cell toxicity by aminoglycosides

Cell suspensions of MDCK cells were incubated with an aminoglycoside (neomycin, gentamicin, and streptomycin) or spermidine, a polyamine for 10 minutes at 37°C followed by the addition of mastoparan (75 ug/ml). At various time points LDH release, ethidium bromide, and fluorescein diacetate uptake were measured to determine membrane permeability and cell viability.

Protein analysis

Aliquots of membrane preparations were digested in 1N NaOH. Protein concentrations were determined by the method of Lowry et al. (1951).

Statistical analysis

Data were analyzed on a Hewlett Packard RT-3 computer using Student's t-test, Paired t-test, Duncan's Multiple Range test and analysis of variance (one way analysis for dose and time studies; two way for combined treatment studies) for comparisons (Snedecor

and Cochran, 1980). Values were considered significantly different at $p < .05$.

Chapter 3:

Mastoparan Mediated Phosphoinositide Metabolism and LDH Release in MDCK cells.

Introduction

Mastoparan has been found to activate phospholipase A_2 activity resulting in the formation of arachidonic acid (Argiolas and Pisano, 1983). In addition, recent studies have shown that mastoparan also activates polyphosphoinositide breakdown (Okano et al., 1985). Though some of the many effects of mastoparan have been studied, the means by which mastoparan causes polyphosphoinositide breakdown has not been well established. Therefore, the effect of mastoparan on phosphoinositide metabolism in MDCK cells was an initial objective.

Since membrane phospholipids are known to regulate membrane permeability, hydrophobic binding of mastoparan to membrane phospholipids may initiate the pathogenesis of injury by altering membrane structure and function. The impairment of the plasma membrane may lead to an increase in membrane permeability which can be quantitated by the leakage of lactate dehydrogenase (LDH: NAD oxidoreductase; EC 1.1.1.27) from the cell. Accordingly, the second objective was to determine whether mastoparan can induce the release of LDH from the cells.

Membrane inositol phospholipid hydrolysis generates an increase in the cytosolic free Ca^{+2} concentration (Hokin, 1985), which may ultimately lead to cell damage or death (Schanne et al., 1979). The mobilization of Ca^{+2} from intracellular stores may be induced by the formation of inositol trisphosphate which is further converted to inositol tetraphosphate and both may act in concert

to induce the influx of Ca^{+2} from the extracellular medium (Burgess et al., 1984). The third objective was to study whether extracellular Ca^{+2} plays a role in modulating the effects of mastoparan on inositol phospholipid hyrolysis and LDH release.

Neomycin, a nephrotoxic polycationic aminoglycoside antibiotic, interacts with polyphosphoinositides (Sastrasinh et al., 1982). It blocks Ca^{++}-dependent histamine secretion from mast cells (Cockcroft et al., 1985), inhibits thrombin-induced proliferation of hamster fibroblasts, and abolishes thrombin-induced inositol phosphate formation in human platelets (Siess and Lepetina, 1986). Since neomycin is a relatively specific inhibitor of inositol phospholipid metabolism (Lang et al., 1977; Lodhi et al., 1979; Lipsky and Lietman, 1982; Marche et al., 1983; Schacht, 1978; Schwertz et al., 1984), and it reacts with negatively charged membrane phospholipids, it may be used to study the role of mastoparan mediated polyphosphoinositide breakdown as a possible mechanism of mastoparan's effect on the cell. Consequently, the final objective was to study the effects of neomycin on mastoparan mediated phosphoinositide hydrolysis and LDH release.

Results

The effect of mastoparan on [^{3}H]inositol phosphates accumulation and [^{3}H]inositol phospholipids depletion in monolayer MDCK cells

The accumulation of [^{3}H]inositol phosphates in MDCK cells was dependent on the concentration of mastoparan. [^{3}H]inositol phosphates increased 56%, 172%, and 231% following treatment of 25, 75, and 100 ug/ml of mastoparan, respectively, (Table 3-1). These effects were also dependent on the duration of exposure to mastoparan (Figure 3-1a). The percent release of [^{3}H]inositol phosphates was 1.8% over basal release at 1 minute ($p<.02$) and peaked at 8% over basal release ($p<.005$) at 15 minutes. Mastoparan depleted [^{3}H]inositol phospholipids by 17% (N.S.) at 1 minute and by 47% at 15 minutes ($p<.001$)(Figure 3-1a).

Effect of mastoparan on lactic dehydrogenase (LDH) release

The release of LDH from MDCK monolayer cells was found to be dependent on the concentration of mastoparan (Table 3-1) and duration of incubation (Figure 3-1b). In mastoparan-treated MDCK cells, LDH release was accompanied by a parallel release of [^{3}H]-inositol phosphates (Table 3-1, Figure 3-1a and 3-1b). LDH release and [^{3}H]-inositol phosphates accumulation in the mastoparan treated MDCK cells were not dependent on external Ca^{++} (Table 3-2).

Effect of mastoparan on [^{3}H]inositol phosphates accumulation and [^{3}H]inositol phospholipid depletion in MDCK cell suspension

In the cultured MDCK cell monolayers, only the apical portion of the cell membrane was exposed to the toxin. Therefore,

isolated MDCK cell suspensions were prepared for the determination of the effect of mastoparan on phosphoinositide metabolism. In the mastoparan-treated cell suspension, the percent net release of [3H]inositol phosphates was measurable at 15 seconds ($p< .02$) and reached 12.5% over basal release by 15 minutes ($p< .001$) (Figure 3-2a).

The response of individual inositol phosphates, i.e. , IP_1, IP_2, and IP_3 to mastoparan was also investigated. An elution profile of these three inositol phosphates is shown in Figure 3-3. Accumulation of IP_2 and IP_3 induced by mastoparan is shown to be time dependent (Figure 3-3). IP_1, IP_2, and IP_3 levels remained constant during the 15 minute incubation period in the untreated cell suspensions (data not shown). At 15 seconds , IP_3 was 180% over basal levels ($p< .006$), and reached a maximum of 421% at 1 minute and declined to 272% at 15 minutes. IP_2 accumulation was not significantly different from control at 15 seconds, but increased to 173% ($p< .04$) at 30 seconds, reaching a maximum of 456% at 15 minutes. IP_1 accumulation was not significantly different from the control at any of the time periods. PI levels showed no signifi-cant changes with time in the mastoparan-treated (75 ug/ml) MDCK cell suspensions (Figure 3-4a). PIP and PIP_2 depleted by 25%, and 50% of control, respectively, at 15 minutes (Figure 3-4b and 3-4c).

Effect of mastoparan on PI and PIP kinase activity in MDCK cell plasma membrane

Increasing concentrations of mastoparan elicited progressive

increases in PI kinase and PIP kinase activity in the plasma membrane enriched fraction "D" (Table 3-3). PI kinase activity, expressed as the amount of PIP formed per 15 ug protein per 10 min, was 44%, 116%, and 167%, at 25, 50, and 75 ug/ml of mastoparan, respectively. In addition, PIP kinase activity, expressed as the amount of PIP_2 formed per 15 ug protein per 10 min, was 142%, 181%, and 226%, at 25, 50, and 75 ug/ml of mastoparan, respectively. The effect was also dependent on the duration of exposure to mastoparan (Table 3-4).

Effects of neomycin on [^{3}H]inositol trisphosphate accumulation and LDH release

The effects of neomycin on IP_3 formation and LDH release in mastoparan-treated MDCK cells were investigated. Neomycin decreased mastoparan-mediated IP_3 formation by 54% and LDH by 49% at 15 seconds (Table 3-5). These effects were dependent on the concentration of neomycin (Table 3-6). Neomycin alone did not affect LDH release in MDCK monolayer cells (data not shown).

Table 3-1. **Effect of mastoparan concentration on [^{3}H]inositol phosphates accumulation and LDH rlelease in MDCK cells.** Cultures were incubated overnight (12-18 hr) with myo-[2-^{3}H]inositol (1.5 uCi/well). Washed cells were incubated with various concentrations of mastoparan for 15 minutes at 37°C. Media were analyzed for LDH (see "Methods"). Inositol phosphates were extracted from the cells and analyzed for radioactivity (see "Methods"). % LDH release = (Actual LDH release/Total LDH) x 100; Total LDH per well = 3248 $\pm$ 200 U/l; Total cells per well = 7.5 X 10^5; p values given when compared to 0 ug/ml mastoparan; values are mean $\pm$ S.E.M.; number of experiments indicated in parenthesis.

Table 3-1: Effect of mastoparan concentration on [^{3}H]inositol phosphate accumulation and LDH release in MDCK cells.

	Mastoparan (ug/ml)						
	0	5	10	25	37.5	75	100
I. [^{3}H]-inositol phosphates [dpm/well]:	752 ±26	825 ±48	912 ±39	990 ±131	1,116 ±32	1,731 ±88	2,103 ±58
	(13)	(6)	(6)	(7)	(6)	(8)	(3)
p values:		p<.05	p<.02	p<.02	p<.001	p<.001	p<.001
II. Actual LDH Release U/l:	103±14	152±15	218±23	494±37	1137±102	1376±42	---
III. % Increase in LDH release:		2%	4%	12%	32%	39%	---
p values:		N.S.	p<.006	p<.001	p<.001	p<.001	---

Table 3-2. **Effect of extracellular Ca^{+2} concentration on [^{3}H]inositol phosphate accumulation and LDH release in mastoparan treated MDCK cells.** Cultures were incubated overnight (12-18 hr) with myo-[2-^{3}H] inositol (1.5 uCi/well). Washed cells were incubated with various extracellular concentrations of calcium with or without mastoparan (75 ug/ml) for 15 minutes at 37°C. The media was analyzed for LDH activity. [^{3}H]inositol phosphates were extracted from the cells and analyzed for radioactivity (see "Methods"). % LDH release = (Actual LDH release/total LDH) X 100; total cells per well = 7.5 X 10^5; values are mean $\pm$ S.E.M.; ( ) = number of experiments; p values:

a. compared to 0 uM Ca^{+2} - mastoparan

b. compared to 100 uM Ca^{+2} - mastoparan

c. compared to 2000 uM Ca^{+2} - mastoparan

d. compared to 0 uM Ca^{+2} + mastoparan

N.S. = not significant ($p>.05$)

Table 3-2. Effect of extracellular Ca^{+2} concentration on 3H-inositol accumulation and LDH release in mastoparan (15ug) treated MDCK cells.

	Concentration of extracellular Ca^{+2} in the presence or absence of 75 ug mastoparan (Toxin)					
	0um Ca^{+2}	0um Ca^{+2} + Toxin	100um Ca^{+2}	100um Ca^{+2} + Toxin	2mM Ca^{+2}	2mM Ca^{+2} + Toxin
I. 3[H]-inositol phosphates [dpm/well]:	432±37 (n=5)	741±53 (n=6)	398±52 (n=7)	813±69 (n=6)	457±36 (n=8)	950±74 (n=8)
p values:		[a]p<.001	[a]N.S.	[b]p<.001 [d]N.S.	[a]N.S.	[c]p<.001 [d]N.S.
II. Actual LDH Release U/l:	116±21 (n=5)	1,117±54 (n=6)	103±17 (n=7)	1,074±81 (n=6)	116±24 (n=8)	1,118±43 (n=8)
p values:		[a]p<.001	[a]N.S.	[b]p<.001 [d]N.S.	[a]N.S.	[c]p<.001 [d]N.S.
III. % LDH release:	3.6%	34%	3.3%	33%	3.6%	34%

Table 3-3. **Effect of mastoparan on phosphoinositide phosphorylation in plasma membranes of MDCK cells.** Fraction "D" (15 ug protein) was preincubated with different concentrations of mastoparan for 5 minutes at 30°C. The reaction was initiated by the addition of 1 mM ATP (final concentration) containing 1.5 uCi (gamma ^{32}P)ATP in the presence of 50 mM Tris-HCl, 20 mM $MgCl_2$, 1 mM EGTA, pH 7.0, and incubated for 10 minutes at 30°C. PIP and PIP_2 levels were quantitated as described in "Methods"; ( ) = number of experiments; values are mean ± S.E.M.; p values: compared to 0 ug/ml mastoparan; PIP = phosphatidylinositol 4-phosphate; PIP_2 = phosphatidylinositol 4,5 bisphosphate.

Table 3-3. Dose response of Mastoparan on Phosphatidylinositol (PI) and phosphatidylinositol phosphate (PIP) kinase activity in purified plasma membranes of cultured MDCK cells.

	Mastoparan Concentration				
	0ug/ml	25ug/ml	50ug/ml	75ug/ml	100ug/ml
	dpm P^{32} / 15ug protein				
PIP^{32}:	117±8.0 (n=8)	168±2.5 (n=4) p<.001	253±14 (n=4) p<.001	312±7.0 (n=4) p<.001	660±26 (n=4) p<.001
PIP_2^{32}	122±13.0 (n=5)	296±35 (n=4) p<.002	343±33 (n=4) p<.001	398±33 (n=4) p<.001	186±13 (n=4) p<.011

Table 3-4. **Time course of mastoparan-mediated phosphoinositide phosphorylation in plasma membranes of MDCK cells.** Fraction "D" (15 ug protein) was incubated with mastoparan (75 ug/ml) for 5 minutes at 30°C. The reaction was initiated by the addition of 1 mM ATP (final concentration) containing 1.5 uCi (gamma^{32}P)-ATP in the presence of 50 mM Tris- HCl, 20 mM $MgCl_2$, 1 mM EGTA, pH 7.0 and incubated at 30°C. At different time intervals PIP and PIP_2 were quantitated as described in "Methods". Number of experiments in each group was four; values are mean $\pm$ S.E.M.; N.S. = not significant; p values compared to 0 minutes.

Table 3-4. Time response of Mastoparan (15ug) on Phosphatidylinositol (PI) and phosphatidylinositol phosphate (PIP) kinase activity in purified plasma membranes of cultured MDCK cells.

	Time in minutes					
	0.0 min.	1 min.	3 min.	5 min.	10 min.	15 min.
Mastoparan (ug/ml)	dpm P^{32} / 15 ug protein					
	PIP^{32}:					
a. 0 ug.:	54±3.8	82±3.0	89±3.7	92±5.2	116±4.0	88±5.0
b. 15 ug.:	61±4.1	141±6.0	181±14	272±10	265±14	204±20
	N.S.	p<.001	p<.001	p<.001	p<.001	p<.002
	PIP_2^{32}:					
a. 0 ug.:	76±2.0	72±7.0	92±10	94±8.0	107±8.0	103±10
b. 15 ug.:	66±2.0	109±10	145±6.6	191±6.8	206±17	189±18
	p<.02	p<.02	p<.005	p<.001	p<.003	p<.007

Table 3-5. **Effect of neomycin on mastoparan-mediated [^{3}H]inositol phosphates accumulation and LDH release in MDCK cells.** Cultures were incubated overnight (12-18 hr) with myo-[^{3}H]inositol (1.5 uCi/well). Washed cells were incubated with mastoparan (75 ug/ml) in the presence or absence of neomycin (2 mM) for appropriate times at 37°C. Media were analyzed for LDH (see "Methods"). Cell extracts were analyzed for IP_1, IP_2, and IP_3 (see "Methods"). Each value for insoitol phosphates represents a pool of four samples. Actual LDH release values are mean ± S.E.M.; % LDH release indicated in brackets = (Actual LDH release/total unstimulated LDH) X 100; Total LDH/well = 3248 ± 200 U/l; ( ) = number of experiments.

Table 3-5: Effect of neomycin concentration on mastoparan-mediated [^{3}H]inositol phosphate accumulation and LDH release in MDCK cells.

	Time		
	0 sec.	10 sec.	15 sec.
I. [^{3}H]inositol phosphates [dpm/well]:			
a. IP_1 (n=3)			
0 mM neomycin:	1,175±264	1,540±260	2,469±423
2 mM neomycin:	1,046±156 N.S.	1,152±169 p<.001	1,718±501 p<.001
b. IP_2 (n=3)			
0 mM neomycin:	340±8.0	710±66	780±63
2 mM neomycin:	349±14 N.S.	476±41 p<.001	490±32 p<.001
c. IP_3 (n=3)			
0 mM neomycin:	372±44	1,026±181	980±56
2 mM neomycin:	360±23 N.S.	494±62 p<.001	571±33 p<.001
II. Actual LDH release U/l:			
0 mM neomycin:	54±2 [1.7%] (n=4)	128±8 [3.9%] (n=8)	321±10 [9.8%] (n=11)
2 mM neomycin:	60±3 [1.8%] (n=7)	65±2 [2.0%] (n=8)	92±6 [2.8%] (n=10)
	N.S.	p<.001	p<.001

Table 3-6. **Effect of neomycin concentration on mastoparan-mediated [^{3}H]inositol phosphate formation and LDH release.** Cultures were incubated overnight (12-18 hr) with myo-[^{3}H]inositol (1.5 uCi/well). Washed cells were incubated with mastoparan (75 ug/ml) in the presence of various concentrations of neomycin for 30 seconds at 37°C. Media were analyzed for LDH (see "Methods"). The cellular extracts were analyzed for IP_1, IP_2, and IP_3 (see "Methods"). Each value represents a pool of four samples. % LDH release = (Actual LDH release/Total LDH) X 100; LDH values are mean ± S.E.M.; ( ) = number of experiments; p values: compared to 0 mM neomycin; [a]total LDH/well = 3248 ± 200 U/l.

Table 3-6. Effect of neomycin concentrations on mastoparan-mediated release of [^{3}H]inositol phosphates and LDH.

	Neomycin(mM)					
	0	.05	0.5	1	1.5	2.0
I. [3H]inositol phosphates [dpm/well]:						
a. IP_1	1713	1553	1538	1628	1646	1339
b. IP_2	432	494	493	398	385	240
c. IP_3	585	579	436	407	371	268
II. Actual LDH Release						
U/l:	321±10	234±12	194±10	165±12	156±14	92±6.0
		p<.001	p<.001	p<.001	p<.001	p<.001
	(4)	(4)	(4)	(4)	(4)	(4)
III.% LDH Release[a]:	10%	7.2%	6%	5%	4.8%	2.8%

Figure 3-1a. **Time course of mastoparan-mediated [^{3}H]inositol phosphates accumulation and [^{3}H]inositol phospholipid depletion.** Cultures were incubated overnight (12-18 hr) with myo-[2-^{3}H]-inositol (1.5 uCi/ml). Washed cells were incubated with various concentrations of mastoparan for 15 minutes at 37°C. Inositol phosphates and inositol phospholipids were extracted from the cells and analyzed for radioactivity (see "Methods"). ( ) = % increase in [^{3}H]inositol phosphates compared to 75 ug/ml mastoparan at time 0. Total cells per well was 7.5 X 10^5. Values are mean $\pm$ S.E.M. p values indicated are compared to 75 ug/ml mastoparan at time 0; * = $p<.05$, ** = $p<.001$.

[3H]inositol phosphates (dpm/well)
[3H]inositol phospholipids (dpm/well)
1600
1400
1200
1000
800
600
400
11000
10000
9000
8000
7000
0
10
20
Time in Minutes
(1.8%)
(5.8%)
(7%)
(8%)
*
**

Figure 3-1b. **Time course of mastoparan-mediated LDH release.** Cultures were washed and incubated with varying concentrations of mastoparan for 15 minutes at 37°C. Media were analyzed for LDH (see "Methods"). Values are mean ± S.E.M. p values indicated are compared to 75 ug/ml mastoparan at time 0; * = $p<.05$, ** = $p<.001$.

Figure 3-2a. **Time course of [^{3}H]inositol phosphate accumulation in mastoparan treated MDCK cell suspensions.** MDCK monolayer cells were incubated overnight (12-18 hr) with myo-[2-^{3}H]inositol (40 uCi/75 cm^2 flask). Cell suspensions were prepared from monolayer culture as described under "Methods" and incubated with mastoparan (75 ug/ml) for appropriate times at 37°C. Cell extracts were analyzed for total [^{3}H]inositol phosphates (see "Methods"). Values are mean ± S.E.M.; each time point was a "pool" of four samples, and each sample contained 7.5 X 10^5 cells; p values indicated were compared to time 0; * = $p<.05$, ** = $p<.001$; ( ) = % release of [^{3}H]inositol phosphates which is expressed as ([^{3}H]inositol phosphates/unstimulated [^{3}H]inositol phospholipids) X 100; total unstimulated [^{3}H]-inositol phospholipid = 70,914 ± 4,327 dpm (n = 4).

Figure 3-2b. **Time course of [^{3}H]inositol monophosphate (IP_1), [^{3}H]inositol bisphosphate (IP_2) and [^{3}H]inositol trisphosphate (IP_3) accumulation in mastoparan treated MDCK cell suspensions.** MDCK monolayer cells were incubated overnight (12-18 hr) with myo-[2-^{3}H]inositol (40 uCi/75 cm^2 flask). Cell suspensions were prepared from monolayer culture as described under "Methods" and incubated with mastoparan (75 ug/ml) for appropriate times at 37°C. Cell extracts were analyzed for IP_1, IP_2, and IP_3 (see "Methods"). Values are mean $\pm$ S.E.M.; each time point was a "pool" of four samples, and each sample contained 7.5 X 10^5 cells; p values indicated are compared to time 0; points without asterisks are not significant ($p>.05$); * = $p<.05$; ** = $p<.001$; IP_1 = [^{3}H]inositol monophosphate; IP_2 = [^{3}H]inositol bisphosphate; and IP_3 = [^{3}H]-inositol trisphosphate.

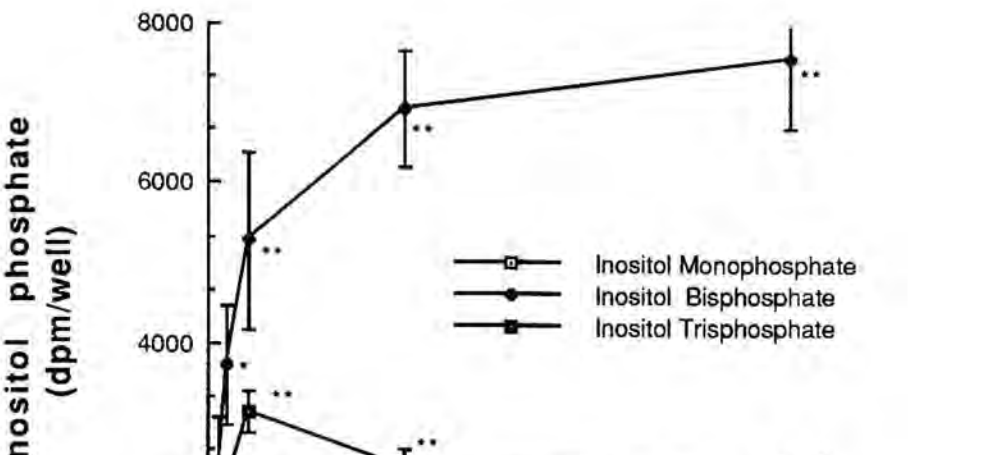

[3H]inositol phosphate (dpm/well)
8000
6000
4000
2000
0
0
10
20
Time In Minutes
Inositol Monophosphate
Inositol Bisphosphate
Inositol Trisphosphate

Figure 3-3. **Elution profile of inositol phosphates.** MDCK cells were incubated with 40 uCi/flask of myo[2-^{3}H]inositol overnight (14-18 hours). Total inositol phosphates were extracted by adding 750 ul chloroform/methanol/4 N HCl (100:200:2, by volume), followed by the addition of 250 ul. chloroform and 250 ul 0.1 N HCl. [^{3}H]inositol monophosphate (IP_1), [^{3}H]inositol bisphosphate (IP_2), and [^{3}H]inositol tris-phosphate (IP_3) were seperated by Dowex 1-X8 anion exchange chromatography as described in "Methods".

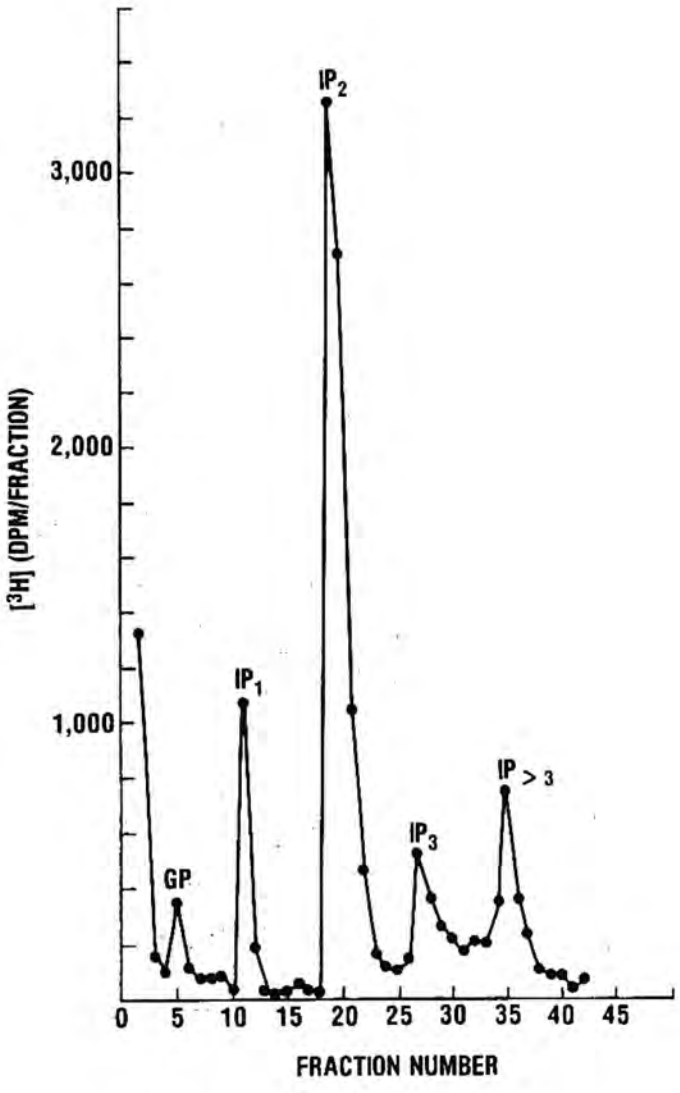

IP2
3,000
2,000
1,000
[3H] (DPM/FRACTION)
IP1
IP > 3
IP3
GP
0
5
10
15
20
25
30
35
40
45
FRACTION NUMBER

Figure 3-4a. **Time course of mastoparan-mediated phosphatidyl-[^{3}H]inositol (PI) formation in isolated MDCK cell suspension.** Cultures were incubated overnight (12-18 hr) with myo-[^{3}H]inositol (1.5 uCi/ml). Cell suspensions were incubated with mastoparan (75 ug/ml). PI (phosphatidylinositol) was extracted and quantitated using procedures described in "Methods". Values are mean $\pm$ S.E.M. for 16 experiments; p values are compared to time 0; N.S. = not significant ($p>.05$); * = $p<.05$.

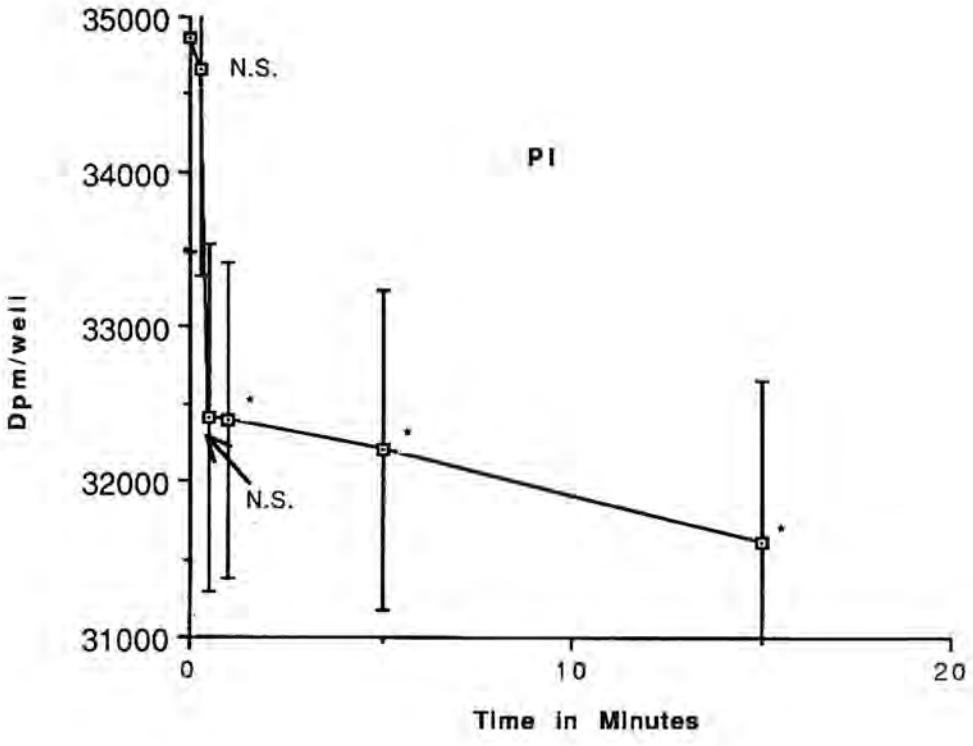


Figure 3-4b. **Time course of mastoparan-mediated phosphatidyl-[^{3}H]inositol 4-phosphate (PIP) formation in isolated MDCK cell suspensions.** Cultures were incubated overnight (12-18 hr) with myo-[^{3}H]inositol 1.5 uCi/ml). Cell suspensions were incubated with mastoparan (75 ug/ml). PIP (phosphatidylinositol 4-phosphate) was extracted and quantitated using procedures described in "Methods". Values are mean $\pm$ S.E.M. for 16 experiments; p values are compared to time 0; N.S. = not significant ($p>.050$); * = $p<.05$; ** = $p<.001$.

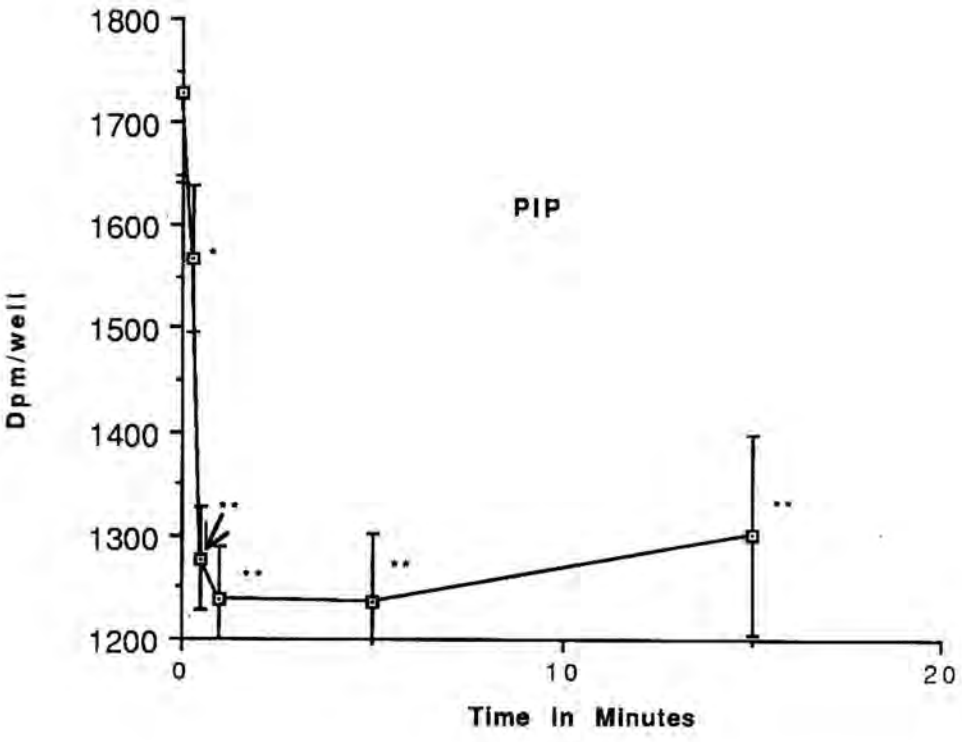
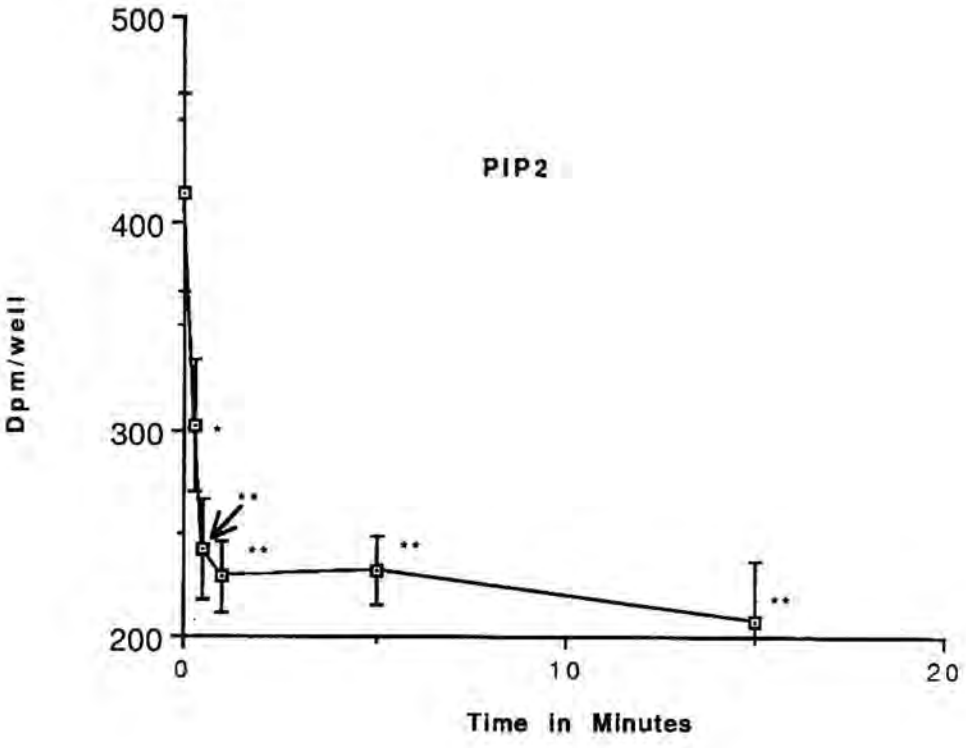


Figure 3-4c. **Time course of mastoparan-mediated phosphatidyl-[^{3}H]inositol 4,5-bisphosphate (PIP_2) formation in isolated cell suspensions.** Cultures were incubated overnight (12-18 hr) with myo-[^{3}H]inositol (1.5 uCi/ml). Cell suspensions were incubated with mastoparan (75 ug/ml). PIP_2 (phosphatidylinositol 4,5-bisphosphate) was extracted and quantitated using procedures described in "Methods". Values are mean $\pm$ S.E.M. for 16 experiments; p values indicated are compared to time 0; N.S. = not significant ($p>.05$); * = $p<.05$; ** = $p<.001$.



Discussion

In this chapter the effect of mastoparan on the accumulation of individual inositol phosphates (IP_1, IP_2, and IP_3) and the hydrolysis of inositol phospholipids (PI, PIP, and PIP_2) was presented. Mastoparan mediated an increase in total inositol phosphate production (Figure 3-2a), of which the percentage increase in both IP_2 and IP_3 production exceeded IP_1 production (Figure 3-2b). No significant changes in mastoparan-mediated IP_1 production were observed within 15 minutes (Figure 3-2b). Furthermore, mastoparan-mediated significant PI, PIP, and PIP_2 depletion within 15 seconds (Figure 3-4a, 3-4b, and 3-4c). Note that the mastoparan-mediated percent release of [^{3}H]-inositol phosphates in the cell suspension (Figure 3-2a) was slightly higher than in the monolayer cells (Figure 3-1a) suggesting that mastoparan may act on both the apical and basolateral membranes.

The continuous accumulation of IP_3 and a sustained depletion of PIP_2 suggest that mastoparan activates phospholipase C. Bacterial originated phospholipase C activity is known to produce cell membrane damage and lead to cell death (Shein, 1982). Since this enzyme has been identified in mammalian cells, it is possible that mastoparan activates phospholipase C in MDCK cells resulting in membrane disruption and, consequently, in the release of LDH. The accumulation of [^{3}H]inositol phosphates occurs concomitantly with the release of LDH (Table 3-1 , Figure 3-1a, and 3-1b).

The possibility that IP_3 accumulation is associated with the release of LDH was also tested. When mastoparan-mediated IP_3 production was reduced by neomycin, mastoparan-mediated LDH release was also reduced (Table 3-5 and 3-6). These results further suggest that IP_3 release is associated with the alteration of membrane function, possibly resulting in cell membrane damage and LDH release.

The influx of extracellular Ca^{++} has been proposed as a final common pathway in toxin-treated cultured liver cell death (Schanne et al., 1979). This finding is, however, contrary to findings obtained with freshly isolated liver cells (Smith et al., 1981). In addition, mastoparan-mediated LDH net release and net production of [^{3}H]inositol phosphates from MDCK cells were not dependent on extracellular Ca^{++} (Table 3-2), although there is a slight increase in mastoparan-induced accumulation of [^{3}H]inositol phosphates in response to increasing Ca^{++} concentrations from 0 mM to 2 mM.

The effect of mastoparan on PI kinase activity in a membrane fraction which appeared to be plasma membrane enriched was also studied. PI kinase has been reported to be localized in the plasma membrane of rat liver (Cockcroft et al., 1985) and human erythrocytes (Garrett and Redman, 1975). The phosphorylation of PI occurred both in the control and in the mastoparan-treated membrane fractions suggesting the toxin has additional effects on PI Kinase (Table 3-3). An increase in mastoparan-mediated PIP_2 levels was also observed (Table 3-3 and 3-4). These observations are in contrast to the results obtained with intact cells in which PIP_2

depletion was observed (Figure 3-4a, 3-4b, and 3-4c). However, it is possible that the time-dependent depletion of PI, PIP and PIP_2 in the mastoparan-treated MDCK cell suspension (Figure 3-4a, 3-4b, and 3-4c) is due to the depletion of intracellular ATP.

In addition to the ability of mastoparan to stimulate phospholipase A_2 (Argiolas and Pisano, 1983), and possibly phospholipase C (Okano et al., 1985), we have demonstrated that mastoparan enhances PI kinase and PIP kinase activities. Further work is required to determine the biochemical mechanisms of action of mastoparan on PI kinase, PIP kinase, and phospholipase C as well as other enzymes involved in the phosphoinositide metabolism.

The results of these studies suggest that mastoparan directly interacts with the membrane resulting in an alteration in phosphoinositide metabolism. The existence of specific receptors for this toxin has not been established. Since membrane phospholipids are known to regulate membrane permeability, hydrophobic binding of mastoparan to membrane phospholipids may initiate the pathogenesis of cell injury by altering membrane structure and function. Consequently, studies are underway to determine the effect of mastoparan on physiological parameters associated with membrane integrity and function.

Chapter 4:

INHIBITION OF ALPHA-AMINOISOBUTYRIC ACID UPTAKE BY MASTOPARAN IN MADIN-DARBY CANINE KIDNEY CELLS

Introduction

The mechanism of cell death involves one or more processes such as energy deprivation, oxidative damage, membrane dissolution, and/or inhibition of protein synthesis (Shier, 1985). Inhibition of protein synthesis due to intracellular amino acid deprivation is a possible mechanism leading to cell death. Since mastoparan is known to alter membrane structure and function a possible mechanism of its lethal effects could be inhibition of amino acid transport. Consequently, the primary objective of the present study was to investigate the effects of mastoparan on the net uptake of Na^+-dependent alpha-aminoisobutyric acid (AIB) in Madin-Darby Canine Kidney (MDCK) culture cells. Mastoparan is known to mediate an increase in polyphosphoinositide breakdown (Okano et al., 1985). By using neomycin, an aminoglycoside antibiotic that has relatively specific inhibitory effects on inositol phospholipid metabolism (Lang et al., 1977; Lodhi et al., 1979; Lipsky and Lietman, 1982; Marche et al., 1983; Schacht, 1978; Schwertz et al., 1984), the second objective was to study the role of mastoparan mediated polyphosphoinositide breakdown as a mechanism for mastoparan's effects on AIB net uptake. Since any changes in AIB net uptake observed could also be due to changes in efflux, the final objective was to determine whether mastoparan or neomycin have any effect on AIB efflux.

Results

Time course of the effects of mastoparan on AIB net uptake

Mastoparan (15 ug/ml) significantly decreased net AIB uptake compared to respective controls at 2.5 minutes ($p<.001$), 5 minutes ($p<.019$), 10 minutes ($p<.019$), and 15 minutes ($p<.001$), (Figure 4-1). Net ^{3}H-AIB uptake by controls was significantly increased at all time points (Figure 4-1). Conversely, there were no significant differences in net ^{3}H-AIB uptake by mastoparan treated cells at any of the time points ($p>.05$), (Figure 4-1).

Dose dependence of the effects of mastoparan on AIB net uptake

Mastoparan produced a dose dependent decrease in net AIB uptake by isolated MDCK cell suspensions. Net AIB uptake was significantly inhibited ($p<.05$) at all mastoparan concentrations tested (Figure 4-2).

Sodium dependence of AIB net uptake

Table 4-1 gives values for AIB net uptake by isolated MDCK cells in the presence of three different extracellular Na^{+} concentrations. An increase in the extracellular Na^{+} concentration correlates with an increase in AIB net uptake.

Effect of ouabain and mastoparan on net ^{3}H-AIB uptake

When isolated cell suspensions were incubated with 1 mM ouabain for 15 minutes, net ^{3}H-AIB uptake was significantly ($p<.05$) decreased by 42% relative to the control values (Figure 4-3). In addition, mastoparan inhibited ^{3}H-AIB transport completely (Figure 4-3).

Effect of neomycin on mastoparan-mediated inhibition of ^{3}H-AIB net uptake

Pre-treatment of isolated MDCK cell suspensions with 2mM neomycin had no significant effect on total ^{3}H-AIB uptake or on ouabain (1 mM) sensitive ^{3}H-AIB uptake (Figure 4-4a). When cells pretreated with neomycin (2mM) were incubated in the presence of mastoparan (75 ug/ml) net ^{3}H-AIB uptake was significantly ($p<.05$) greater relative to that of cells treated with mastoparan (75ug/ml) alone (Figure 4-4 a,b). However, uptake by the cells treated with mastoparan and pre-treated with neomycin remained 39% (significantly; $p<.05$) lower than control values.(Figure 4-4 a,b).

Effect of neomycin on mastoparan-induced ^{3}H-AIB efflux

Mastoparan-mediated increases in ^{3}H-AIB efflux were inhibited by neomycin in a dose (Figure 4-5) and time dependent fashion (Figure 4-6). Complete inhibition of mastoparan-augmented efflux occurred at a neomycin concentration of 2mM (Figure 4-5). At 2mM neomycin, the mastoparan-mediated increase in ^{3}H-AIB efflux was reduced to control levels (Figure 4-6), and the amount of ^{3}H-AIB effluxed was not significantly different ($p> .05$) from that of the control at each of the respective time points (Figure 4-6). Neomycin treatment alone did not significantly affect ($p> .05$) efflux relative to that of the respective controls at any of the time points (Figure 4-6).

Table 4-1. **Sodium dependence of [^{3}H]AIB net uptake by isolated cell suspensions of MDCK cells.** Solutions A and B were formulated with the same components as Dulbecco's Phosphate Buffered Saline except that choline chloride was substituted for the missing portion of NaCl. Solution C was Dulbecco's Phosphate Buffered Saline. Values are mean ± S.E.M. Number of experiments is given in parenthesis.

*Concentrations of Na^+ and K^+ were determined with a Beckman Klina-Flame Spectrophotomter.

**Concentrations of Cl^- were determined with a Radiometer-Copenhagen CMT 10 Cl^- Titrator.

***Osmolarities of the solutions were determined with an Advanced Instrument Digitmatic Osometer 3D-II.

[a]$p<.001$ compared to A.

Table 4-1. Sodium dependence of [^{3}H]-AIB net uptake by isolated cell suspensions of MDCK cells.

SOLUTION	Na^+* (mEq/l)	K^+* (mEq/l)	Cl^-* (mEq/l)	Osmolarity*** (mOsm/l)	pH	3H-AIB Uptake (nmol/mg protein/ 15 min.)	
A	28.5	4.0	139	289	7.4	93 ± 3.0	(9)
B	96	3.6	138	291	7.4	115 ± 4.0[a]	(9)
C	155	4.2	144	287	7.5	132 ± 5.6[a]	(6)

Figure 4-1. **Effect of mastoparan on [^{3}H]AIB net uptake in MDCK cells.** Isolated MDCK cell suspensions were incubated with [^{3}H]AIB at 37°C in either phosphate buffered saline (PBS) alone (control) or in PBS containing mastoparan (75 ug/ml) for the times indicated. [^{3}H]AIB uptake was determined as described in "Methods". Values are the means ± S.E.M. The number of experiments is given in parenthe-sis. p values are compared to time 0; N.S. = not significant (p>.05); * = p<.05; ** = p<.001.

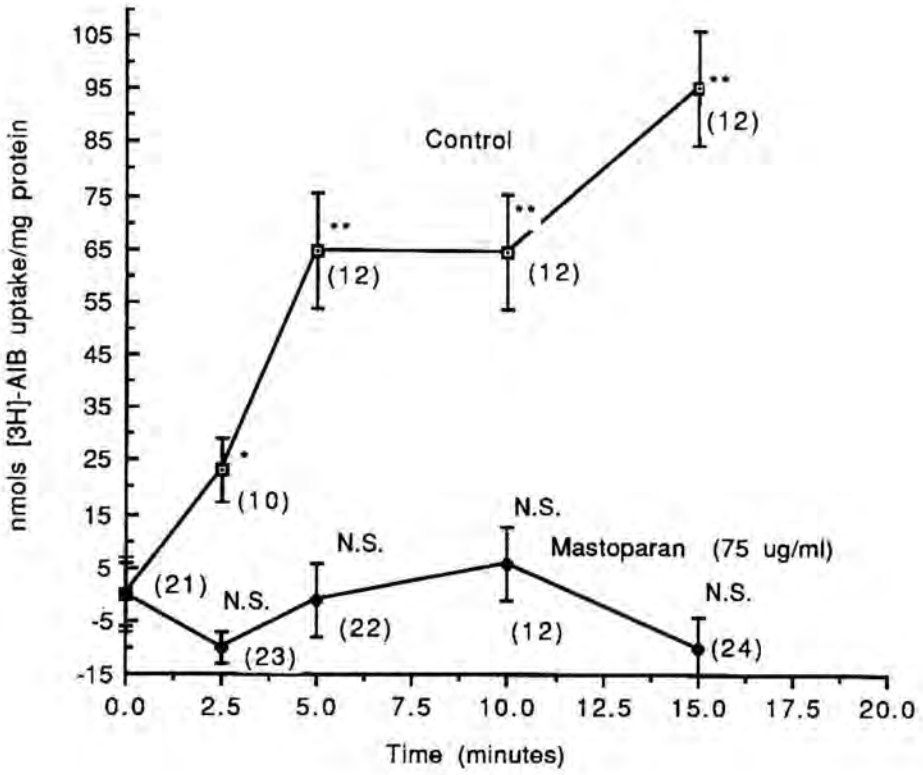

nmols [3H]-AIB uptake/mg protein
105
95
85
75
65
55
45
35
25
15
5
-5
-15
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Time (minutes)
Control
Mastoparan (75 ug/ml)
(21)
(10)
(12)
(12)
(12)
(22)
(23)
(12)
(24)
N.S.
N.S.
N.S.
N.S.
*
**
**
**

Figure 4-2. **Dose response of mastoparan on [^{3}H]AIB uptake in MDCK cells.** Isolated MDCK cell suspensions were incubated with [^{3}H]AIB at 37°C for 15 minutes in PBS containing the concentrations of mastoparan indicated. [^{3}H]AIB net uptake was determined as described in "Methods". Values presented are the means ± S.E.M. The number of experiments are given in brackets. p values indicated are compared to 0 ug/ml mastoparan; ** = p<.001.

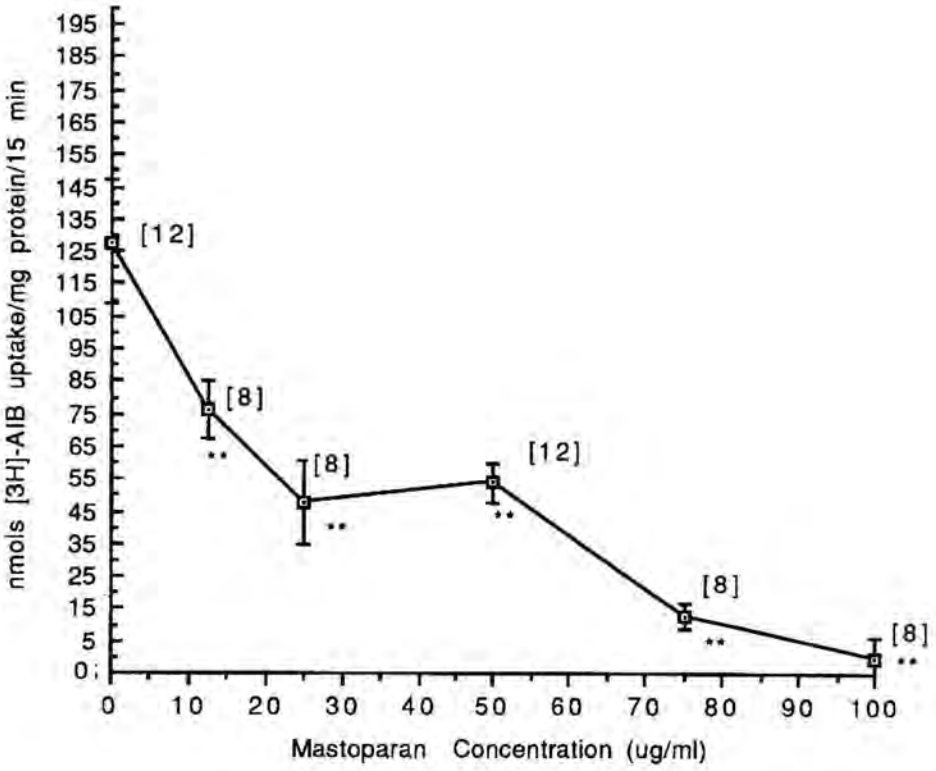

nmols [3H]-AIB uptake/mg protein/15 min
195
185
175
165
155
145
135
125
115
105
95
85
75
65
55
45
35
25
15
5
0
[12]
[8]
[8]
[12]
[8]
[8]
**
**
**
**
**
**
0
10
20
30
40
50
60
70
80
90
100
Mastoparan Concentration (ug/ml)

Figure 4-3. **Effects of ouabain and mastoparan on [^{3}H]AIB uptake** Isolated MDCK cell suspensions were incubated with [^{3}H]AIB at 37°C for 15 minutes in PBS alone (control) or in PBS containing ouabain or mastoparan at the concentrations indicated in parentheses. This mixture was pulsed for 15 minutes at 37°C and the amount of [^{3}H]AIB was quantitated using procedures described in "Methods". Values are the mean ± S.E.M. The number of experiments are in brackets. p values indicated are compared to control uptakes; ** = p<.001.

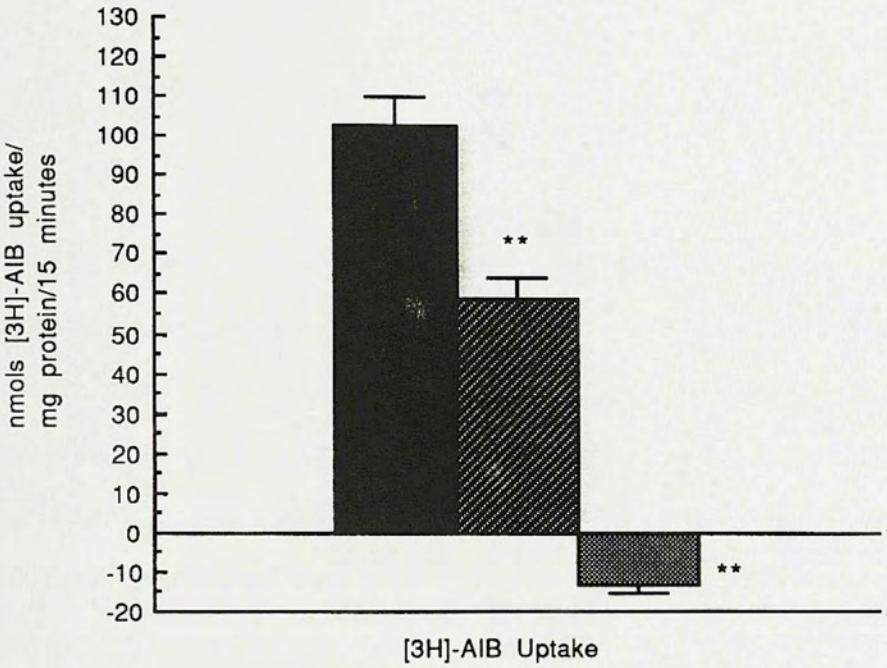

130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
nmols [3H]-AIB uptake/
mg protein/15 minutes
**
**
[3H]-AIB Uptake
Control [24]
Ouabain (1X10 -3 M) [26]
Mastoparan (75 ug/ml) [18]

Figure 4-4a. **Time course of the effects of neomycin on [^{3}H]AIB uptake in MDCK cells.** Isolated MDCK cell suspensions of MDCK cells were incubated with [^{3}H]AIB at 37°C for the times indicated in PBS alone (control) or in PBS containing neomycin, mastoparan, or mastoparan + neomycin at the concentrations indicated in parentheses. [^{3}H]AIB uptake was determined as described in "Methods". Values are the mean ± S.E.M. The number of experiments are in brackets. p values indicated are compared to time 0; N.S. = not significant ($p>.05$); * = $p<.05$; ** = $p<.001$.

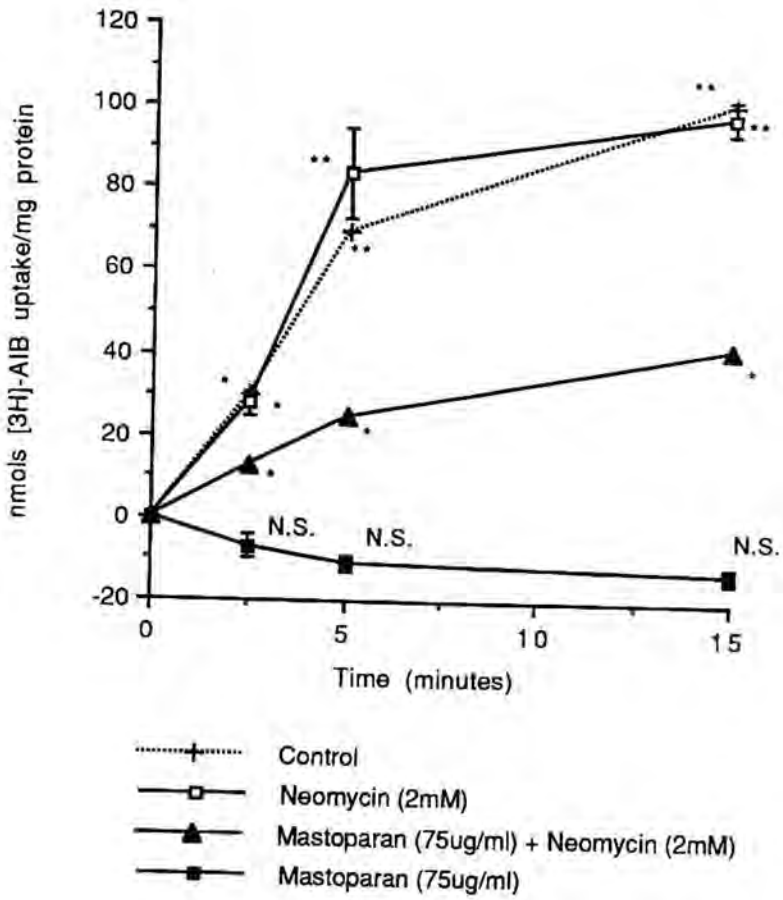

120
100
80
60
40
20
0
-20
nmols [3H]-AIB uptake/mg protein
N.S.
N.S.
N.S.
0
5
10
15
Time (minutes)
Control
Neomycin (2mM)
Mastoparan (75ug/ml) + Neomycin (2mM)
Mastoparan (75ug/ml)

Figure 4-4b. **Effects of neomycin and ouabain on [^{3}H]AIB net uptake** Isolated MDCK cell suspensions of MDCK cells were incubated with [^{3}H]AIB at 37°C for 15 minutes in PBS alone (control) or in PBS containing neomycin, mastoparan, ouabain, mastoparan and neomycin or ouabain and neomycin at the concentrations indicated in parentheses. [^{3}H]AIB uptake was determined as described in "Methods". Values are the mean ± S.E.M. The number of experiments are in brackets. p values indicated are compared to controls; N.S. = not significant ($p>.05$); * = $p<.05$; ** = $p<.001$.

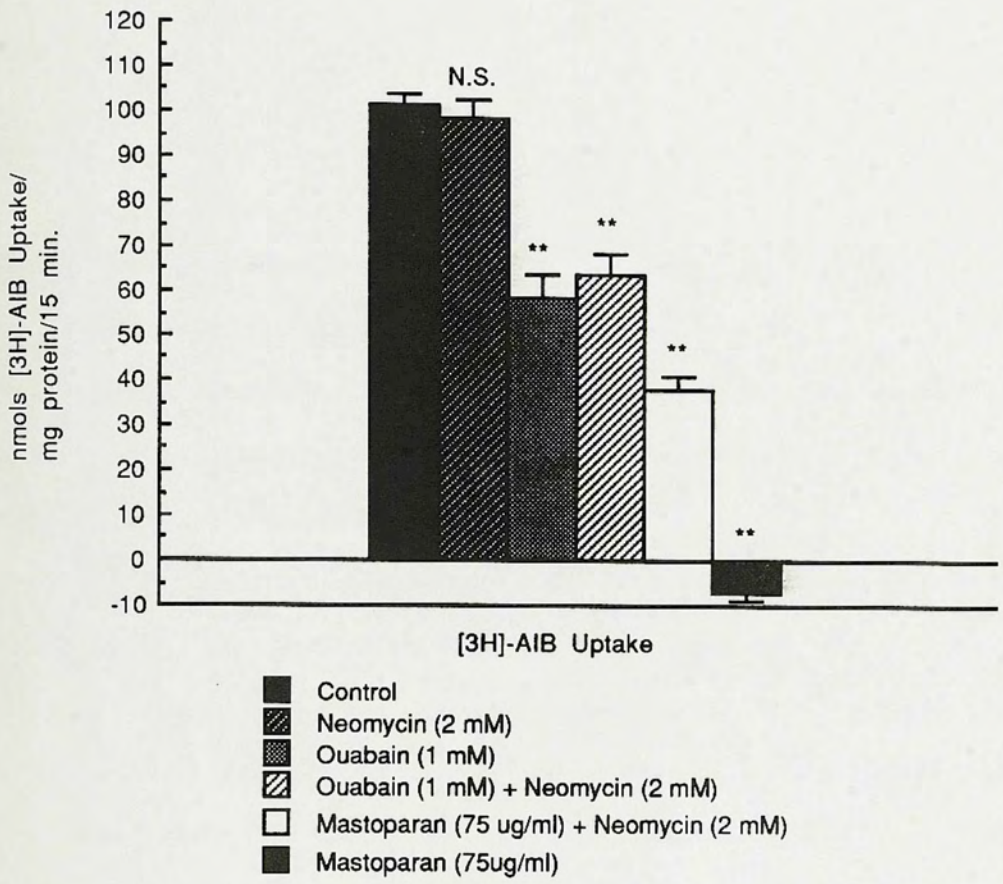

120
110
100
90
80
70
60
50
40
30
20
10
0
-10
nmols [3H]-AIB Uptake/
mg protein/15 min.
N.S.
**
**
**
**
[3H]-AIB Uptake
Control
Neomycin (2 mM)
Ouabain (1 mM)
Ouabain (1 mM) + Neomycin (2 mM)
Mastoparan (75 ug/ml) + Neomycin (2 mM)
Mastoparan (75ug/ml)

Figure 4-5. **Dose response of neomycin on mastoparan-mediated [^{3}H]AIB efflux.** Aliquots of isolated MDCK cell suspensions were loaded with [^{3}H]AIB as described in "Methods". After loading, the cells were pre-incubated in PBS containing various concentrations of neomycin for 5 minutes at 37°C. Upon completion, mastoparan was added to each aliquot to give a final concentration of 75 ug/ml and efflux from the "loaded" cells was determined at 37°C for 15 minutes as described in "Methods". Values are the means ± S.E.M. The number of experiments was six (6). p values indicated are compared to controls at each respective time point; N.S. = not significant; * = $p<.05$; ** = $p<.001$.

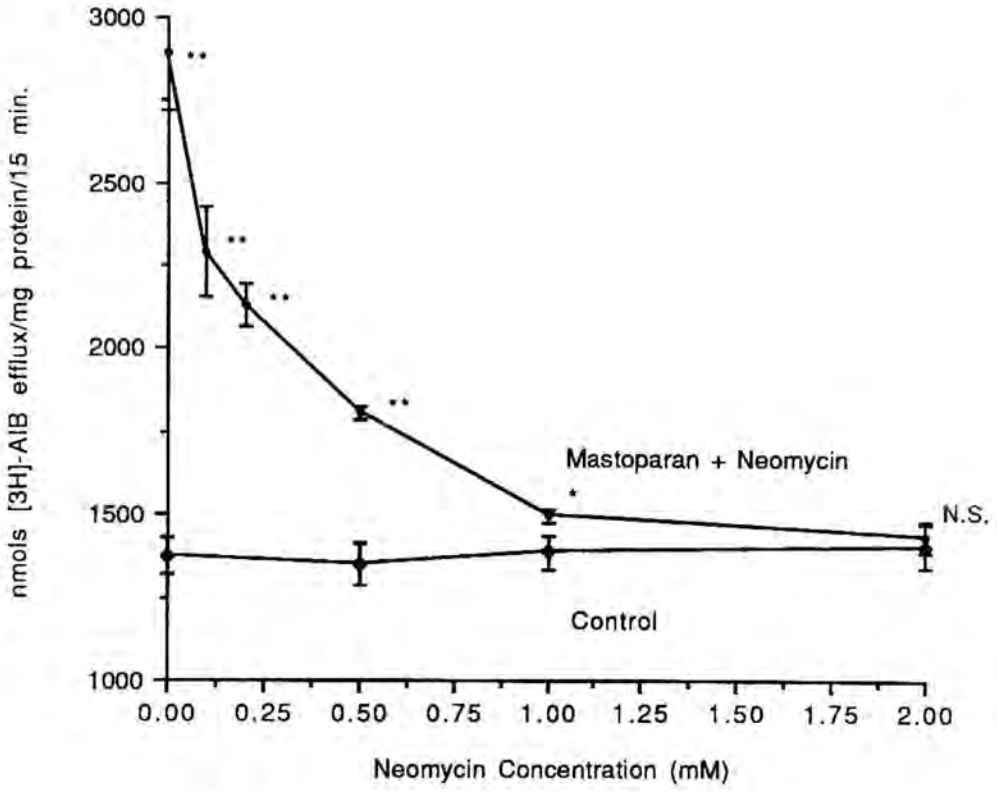
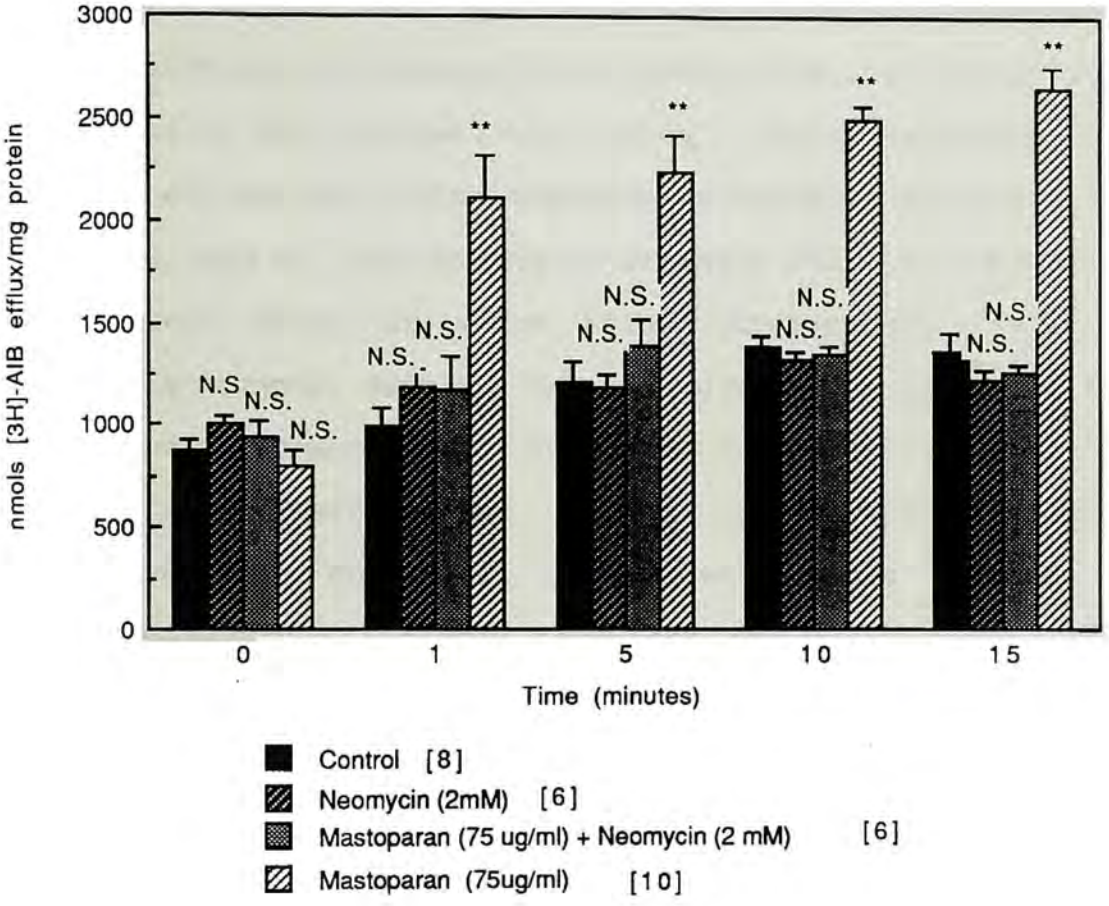

3000
2500
2000
1500
1000
nmols [3H]-AIB efflux/mg protein/15 min.
**
**
**
**
*
Mastoparan + Neomycin
N.S.
Control
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
Neomycin Concentration (mM)

Figure 4-6. **Effect of neomycin on mastoparan-mediated [^{3}H]AIB efflux.** Aliquots of MDCK cell suspensions were loaded with ^{3}H-AIB as described in "Methods". A 180 ul aliquot of this suspension was treated with mastoparan (15 ug/ml) and/or neomycin (2 mM) in 20 ul. The rate of efflux was determined as described in "Methods" for the various time points and conditions indicated. Values are the mean ± S.E.M. Number of experiments is indicated in brackets. p values indicated are compared to control values at each respective time point; N.S. = not significant ($p>.05$); ** = $p<.001$.


3000
2500
2000
1500
1000
500
0
nmols [3H]-AIB efflux/mg protein
N.S
N.S.
N.S.
N.S.
N.S.
N.S.
N.S.
N.S.
N.S.
N.S.
N.S.
N.S.
N.S.
**
**
**
**
0
1
5
10
15
Time (minutes)
Control [8]
Neomycin (2mM) [6]
Mastoparan (75 ug/ml) + Neomycin (2 mM) [6]
Mastoparan (75ug/ml) [10]

Discussion

Amino acid transport is essential for the maintenance of cellular metabolism. Impairment of amino acid transport can result in depletion of intracellular amino acids, inhibition of protein synthesis, and eventual cell death. Active transport of amino acids across the plasma membrane is mediated primarily by systems A, ASC, and L, each having an affinity which is specific for a different class of amino acids (Christensen, 1975). Alpha-aminoisobutyric acid, a non-hydrolyzable amino acid analog, is transported specifically by system A. It was used in the present study to determine the effects of mastoparan on amino acid transport in MDCK cell suspensions. The results show that mastoparan significantly decreases net AIB uptake in a time and dose dependent fashion (Figure 4-1 and Figure 4-2).

System A transport is energized, in part, by the electrochemical gradient of Na^+ across the plasma membrane (Christensen and Handlogten, 1977). When the concentration of sodium in the medium was raised from 28 mEq/l to 155 mEq/l, AIB net uptake increased 42% (Table 4-1), suggesting that AIB uptake in MDCK cells is Na^+ dependent. Ouabain (1 mM), a specific inhibitor of Na^+K^+-ATPase activity, was found to inhibit AIB net uptake (Figure 4-3). These observations suggest that a relationship exists between Na^+,K^+-ATPase activity, the Na^+ gradient, and the rate of AIB transport. As described previously, changes in Na^+,K^+-ATPase activity precede changes in AIB net uptake during treatment with

triiodothyronine, implying that AIB is probably mediated by Na^+,K^+-ATPase activity (Eng and Lo, 1987). Since Na^+,K^+-ATPase activity is the energy source for the development and maintenance of the Na^+ gradient, it is possible that mastoparan inhibits AIB uptake by inhibiting Na^+,K^+-ATPase activity (Chapter 5). We have found, however, that inhibition of AIB uptake does not appear to be due entirely to inhibition of Na,K-ATPase activity. In the presence of 1 mM Ouabain, AIB net uptake was inhibited by only 36%, whereas mastoparan (75 ug/ml) completely inhibited net AIB uptake (Figure 4-3). Since 1 mM ouabain is sufficient to completely inhibit Na^+,K^+-ATPase activity, this additional inhibition by mastoparan must be due to some mechanism(s) other than inhibition of Na^+,K^+-ATPase activity. A possible mechanism is that mastoparan causes an increase in membrane permeability resulting in the dissipation of the Na^+ gradient and inhibition of amino acid uptake by the cells. An increase in membrane permeability to AIB could also explain the mastoparan mediated increase in AIB efflux.

Mastoparan is known to mediate the accumulation of inositol trisphosphate with a concomitant decrease in the levels of phosphatidylinositol 4,5-bisphosphate by activating phospholipase C activity (Okano et al., 1985). The possibility that the phosphoinositide metabolism is involved in mastoparan mediated increases in AIB efflux is suggested by the following observations. Neomycin (2mM), an inhibitor of inositol phospholipid metabolism (Lang et al., 1977; Lodhi et al., 1979; Lipsky and Lietman, 1982; Marche et al., 1983; Schacht, 1978; Schwertz et al., 1984),

completely suppressed the AIB efflux induced by mastoparan (Figure 4-5 and 4-6). Since 1 mM ouabain was present in the incubation medium, neomycin's inhibition of mastoparan-induced AIB efflux is not likely to involve the reversal of mastoparan induced Na^+,K^+-ATPase inhibition. However, the same concentration of neomycin only partially overcame the inhibition of AIB uptake induced by mastoparan (Figure 4-4) suggesting that a mechanism, in addition to stimulation of phospholipase C activity, plays a role in the inhibition of AIB uptake by mastoparan. Other possible mechanisms include inhibition of Na^+,K^+-ATPase as discussed above.

The present data do not rule out the possibility that other mechanisms may be involved in the inhibition of AIB net uptake by mastoparan. It is possible that mastoparan inhibits AIB net uptake by inhibiting amino acid carriers directly or by inhibiting oxidative phosphorylation resulting in adenosine trisphosphate (ATP) depletion and a loss of the Na^+ gradient. The present data do suggest that mastoparan is involved in the impairment of AIB uptake and enhancement of AIB efflux in MDCK cells. Both of these effects could deplete the intracellular content of amino acids and eventually lead to cell death.

Chapter 5:

INHIBITION OF RAT RENAL NA^+,K^+-ATPase BY MASTOPARAN

Introduction

The changes in amino acid transport observed previously may be due to changes in Na^+,K^+-ATPase activity since AIB net uptake is mediated in part by Na^+,K^+-ATPase activity (Eng and Lo, 1987). Based on evidence that mastoparan activates phosphatidylinositol specific phospholipase C (Okano et al., 1985) and that phosphatidylinositol is required for activation of the Na^+,K^+-ATPase in microsomal membranes prepared from rat kidney (Mandersloot et al., 1978; Roelofsen and VanLindeSibenius, 1981), the primary objective of the present study was to determine the effects of mastoparan on Na^+,K^+-ATPase. In prelminary experiments we found that mastoparan inhibits rat renal Na^+,K^+-ATPase activity. A more detailed study was therefore performed to determine the effects of mastoparan on the Vmax of rat kidney Na^+,K^+-ATPase and on the Kd (apparent dissociation constant) for Na^+, K^+, and ATP.

Mastoparan could inhibit Na^+,K^+-ATPase by reducing the number of enzyme sites, or by decreasing the rate of turnover of each site. Either of these mechanisms would result in a decrease in the Vmax of the enzyme. The second objective, therefore, was to determine the effect of mastoparan on the generation of phosphorylated intermediate. If the same amount of phosphorylated intermediate were formed in the presence of mastoparan as in its absence, then this would suggest that mastoparan inhibits by reducing the turnover rather than the number of sites.

Alternatively, if the inhibition of Na^+,K^+-ATPase was mediated through the degradation of the phosphatidylinositol "pool", then the treatment of microsomal membranes with neomycin, an aminoglycoside antibiotic that is a relatively specific inhibitor of inositol phospholipid metabolism (Lang et al., 1977; Lodhi et al., 1979; Lipsky and Lietman, 1982; Marche et al., 1983; Schacht, 1978; Schwertz et al., 1984), should abolish or attenuate the inhibition of Na^+,K^+-ATPase by mastoparan. The final objective, therefore, was to determine the effects of neomycin on inhibition of Na^+,K^+-ATPase by mastoparan.

Results

Time course of the effects of mastoparan on Na^+, K^+-ATPase activity

Mastoparan (75 ug/ml) significantly decreased ($p<.05$) Na^+,K^+-ATPase activity compared to the control (assayed in the absence of mastoparan) at all time points (Figure 5-1). At this dosage a maximal inhibition of 18% was achieved in 30 seconds (Figure 5-1).

Dose dependence of the effect of mastoparan on Na^+, K^+-ATPase activity

Mastoparan produced a dose dependent decrease in rat kidney Na^+,K^+-ATPase activity (Figure 5-2). Na^+,K^+-ATPase activity was significantly inhibited ($p<.05$) at all mastoparan concentrations tested, and at the lowest (12.5 ug/ml) and highest (100 ug/ml) concentrations the inhibition was 12% and 24%, respectively (Figure 5-2).

The effect of mastoparan on the kinetics of activation of Na^+, K^+-ATPase by sodium, potassium, and ATP

The pattern of activation by sodium of rat kidney Na^+,K^+-ATPase treated with mastoparan (75 ug/ml) and that of controls is shown in Figure 5-3. The Kd's for Na^+ obtained from Hill plots (Figure 5-3, inset) were 15.28 ± 0.89 mM for the controls and 16.74 ± 0.96 mM for the mastoparan treated membranes ($n=6$, $p \geq .577$). The Hill coefficients (an index of the number of binding sites and their degree of interaction) were 1.60 ± 0.09 and 1.37 ± 0.14 for the controls and mastoparan treated membranes, respectively ($n=6$, $p \geq .322$), (Figure 5-3, inset).

The pattern of activation by K^+ of rat kidney Na^+,K^+-ATPase treated with mastoparan and that of the controls is shown in Figure 5-4. A significant decrease from a control Kd of 1.03 ± 0.07 mM to a Kd value of 0.51 ± 0.041 mM for the mastoparan treated membranes was observed (n=6, $p<.003$) (Figure 5-4, inset). The Hill coefficients for Na^+ were 0.82 ± 0.05 for the controls and 1.0 ± 0.06 for the mastoparan treated membranes (n=4, $p<.031$), (Figure 5-4, inset).

The pattern of activation by ATP of rat kidney Na^+,K^+-ATPase treated with mastoparan and that of controls is shown in Figure 5-5. The Kd's for ATP were 0.41 ± 0.10 mM for the controls and 0.35 ± 0.10 mM for the mastoparan treated membranes (n=6, $p\geq.059$) (Figure 5-5, inset). The Hill coefficients were 2.20 ± 0.29 and 2.69 ± 0.69 for the controls and mastoparan treated membranes, respectively (n=6, $p\geq.551$) (Figure 5-5, inset).

The effect of mastoparan on the formation of phosphorylated intermediate

The amount of phosphorylated intermediate per mg protein was 21.5% greater in the mastoparan treated membranes than in the controls ($p<.001$)(Figure 5-6).

The effect of neomycin on the inhibition of Na^+,K^+-ATPase by mastoparan

Pre-treatment of the membranes with 2 mM neomycin had no significant effect on Na^+,K^+-ATPase activity (data not shown). When the membranes were pre-treated with varying concentrations of

neomycin and incubated in the presence of mastoparan (75 ug/ml), Na^+,K^+-ATPase activity was greater ($p < .05$) compared to that of membranes treated with mastoparan (75 ug/ml) alone (Table 5-1).

Table 5-1. **Effect of neomycin on rat renal Na^+,K^+-ATPase activity treated with mastoparan.** Controls represent membrane preparations that have no treatment. Other membrane preparations were pre-treated with varying concentrations of neomycin then treated with mastoparan (75 ug/ml). Na^+,K^+-ATPase activity was determined as described in "Methods". Activity is expressed as umoles Pi per mg protein per hour. Values represent the mean $\pm$ S.E.M. of the number of experiments indicated. p values given are compared to controls.

Table 5-1. Effect of neomycin on rat renal Na^+,K^+-ATPase treated with mastoparan:

Control	Mastoparan Treated				
	Concentrations of Neomycin				
	0mM	0.25mM	0.50mM	1.0mM	2mM
Na^+,K^+-ATPase Activity (umoles Pi/mg protein/hr):					
52±1.6	46±1.4	47.4±1.7	47.6±1.3	48.5±1.3	50.5±1.5
n=10	n=8	n=6	n=6	n=6	n=6
	p<.05	p<.05	p<.05	p<.05	N.S.

Figure 5-1. **Time course of the effects of mastoparan on rat renal Na^+,K^+-ATPase activity.** Membrane preparations were treated with mastoparan (75ug/ml) for various times. Na^+,K^+-ATPase activity was measured as described in "Methods". Values are mean ± S.E.M. (n=9). p values indicated are compared to time 0; ** = p<.001

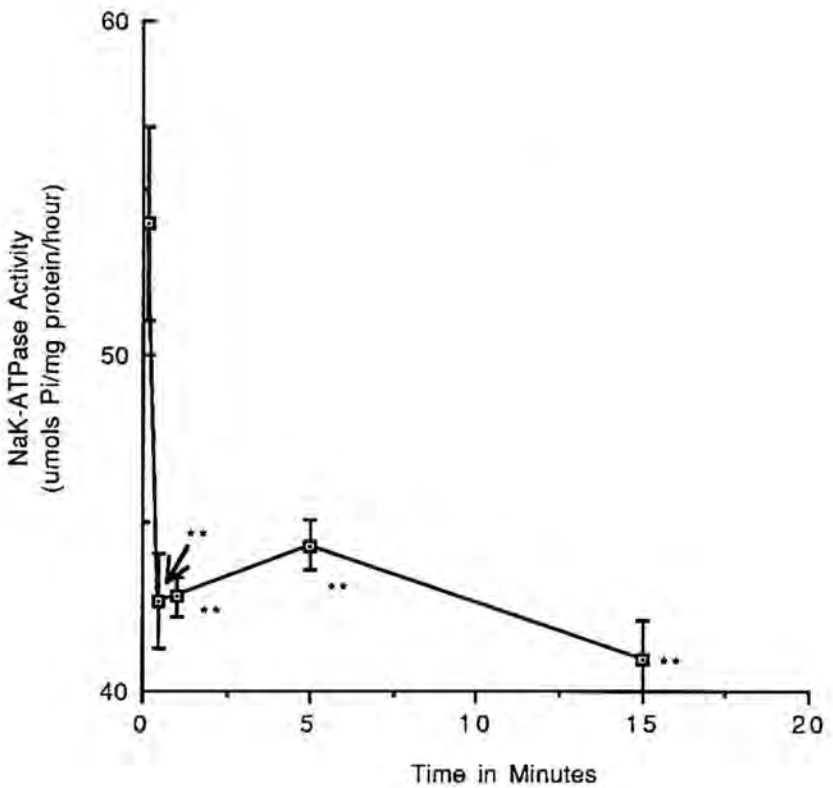

60
50
40
0
5
10
15
20
NaK-ATPase Activity
(umols Pi/mg protein/hour)
Time in Minutes
**
**
**
**

Figure 5-2. **Dose dependence of the effects of mastoparan on rat renal Na^+,K^+-ATPase activity.** Membrane preparations were treated with varying concentrations of mastoparan. Na^+,K^+-ATPase was determined as described in "Methods". Values presented are mean ± S.E.M. (n=10). p values indicated are compared to 0 ug/ml mastoparan; ** = p<.001

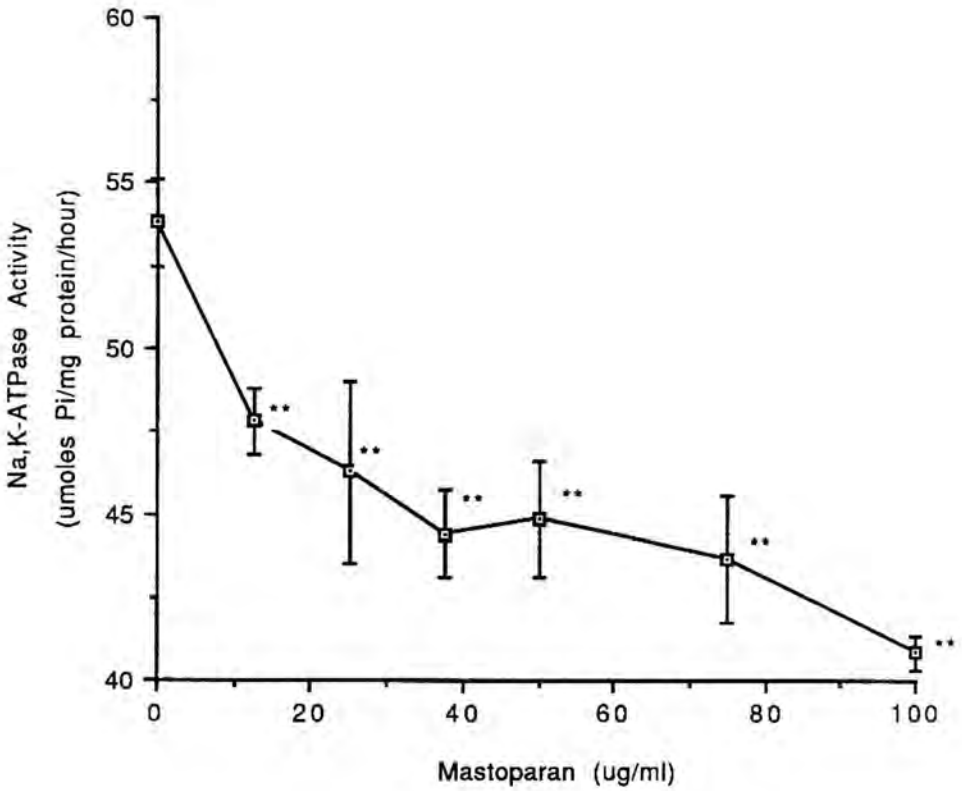

Na,K-ATPase Activity
(umoles Pi/mg protein/hour)
60
55
50
45
40
0
20
40
60
80
100
Mastoparan (ug/ml)
**
**
**
**
**
**

Figure 5-3. **Pattern of activation by sodium of rat renal Na^+,K^+-ATPase.** Membrane preparations were treated with distilled water (□) or distilled water containing 75 ug/ml mastoparan (■) and activated by varying concentrations of Na^+. Each point represents the mean ± S.E.M. (n=6). p values indicated are compared to control values at each respective sodium concentration; * = $p<.05$; all other values are not significant ($p>.05$). Inset: Hill Plot of data in Figure 3. K_D (apparent dissociation constant for Na^+) was determined as the antilog of the value on the x axis when y = 0. The Hill coefficient was obtained from the slope. Each value represents the mean ± S.E.M. (n = 6). Lines were drawn by linear regression.

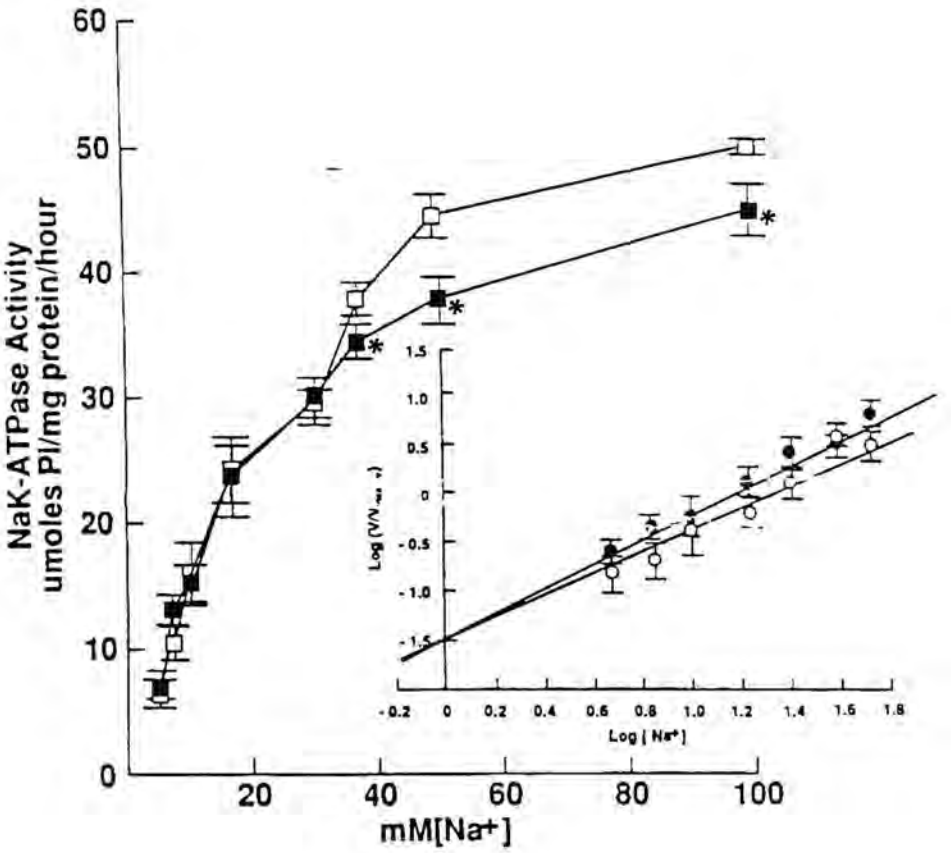

NaK-ATPase Activity
umoles Pi/mg protein/hour
mM[Na+]
Log [Na+]

Figure 5-4. **Pattern of activation by potassium of rat renal Na^+,K^+-ATPase.** Membrane preparations were treated with distilled water (□) or distilled water containing 75 ug/ml mastoparan (■) and activated by varying concentrations of K^+. Each point represents the mean ± S.E.M. (n=8). p values indicated are compared to control values at each respective potassium concentration; * = $p<.05$; all other values are not significant ($p>.05$). Inset: Hill Plot of data in Figure 5-4. K_0 (apparent dissociation constant for K^+) was determined as the antilog of the value on the x axis when y = 0. The Hill coefficient was obtained from the slope. Each value represents the mean ± S.E.M. (n = 6). Lines were drawn by linear regression.

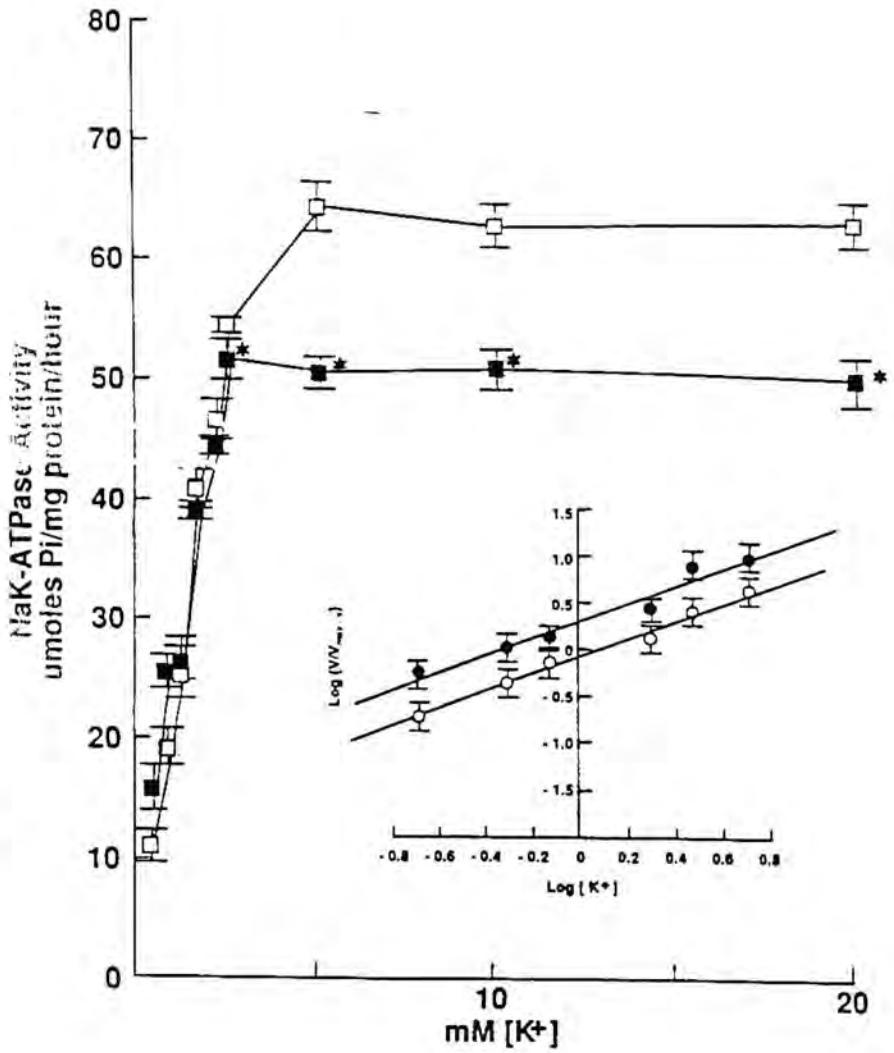

80
70
60
50
40
30
20
10
0
umoles Pi/mg protein/hour
10
20
mM [K+]
1.5
1.0
0.5
0
- 0.5
- 1.0
- 1.5
- 0.8
- 0.6
- 0.4
- 0.2
0
0.2
0.4
0.6
0.8
Log [K+]

Figure 5-5. **Pattern of activation by ATP of rat renal Na^+,K^+-ATPase.** Membrane preparations were treated with distilled water (□) or distilled water containing 75 ug/ml mastoparan (■) and activated by varying concentrations of ATP. Each point represents the mean ± S.E.M. (n=9). p values indicated are compared to control values at each respective ATP concentration; * = $p<.05$; all other values are not significant ($p>.05$). Inset: Hill Plot of data in Figure 5-5. K_D (apparent dissociation constant for ATP) was determined as the antilog of the value on the x axis when y = 0. The Hill coefficient was obtained from the slope. Each value represents the mean ± S.E.M. (n = 6). Lines were drawn by linear regression.

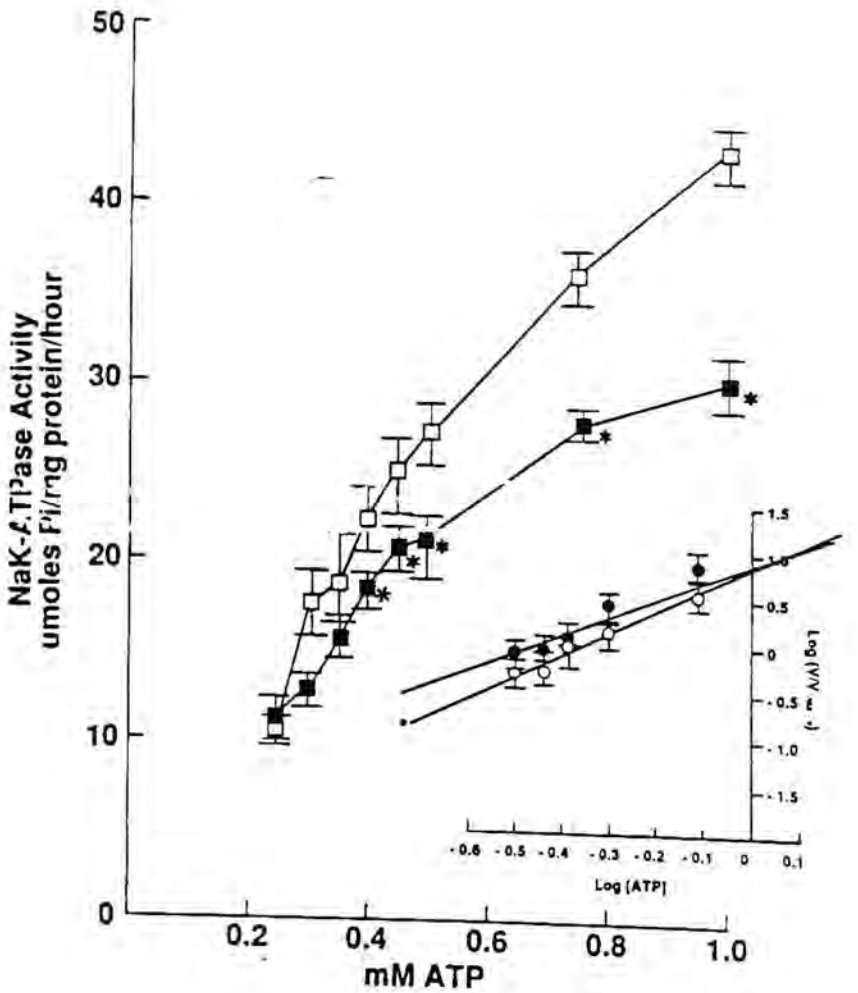
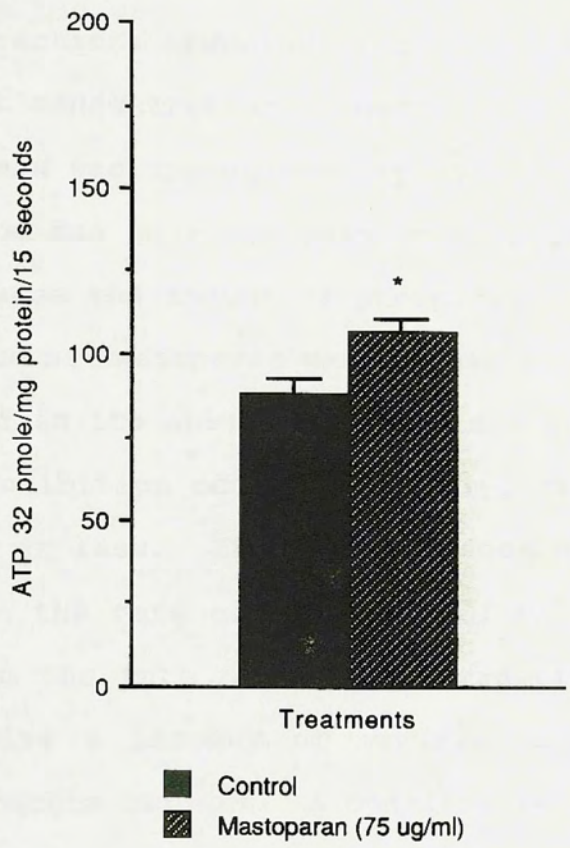

NaK-ATPase Activity
umoles Pi/mg protein/hour
50
40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
mM ATP
1.5
1.0
0.5
0
- 0.5
- 1.0
- 1.5
- 0.6 - 0.5 - 0.4 - 0.3 - 0.2 - 0.1 0 0.1
Log [ATP]

Figure 5-6. **Effect of mastoparan on the formation of phosphorylated intermediate.** Membrane preparations were either treated with diluent or mastoparan (75 ug/ml). Generation of phosphorylated intermediate is described in "Methods". Value are mean ± S.E.M. n = 16. * = value significantly different from controls ($p<.05$).


200
150
100
50
0
ATP 32 pmole/mg protein/15 seconds
*
Treatments
Control
Mastoparan (75 ug/ml)

Discussion

In the present study, mastoparan was found to produce a concentration dependent inhibition of Na^+,K^+-ATPase activity of membrane fractions prepared from rat kidney. At 100 ug/ml, the highest concentration of mastoparan tested, inhibition of the enzyme's Vmax was approxiamately 25%. The inhibition does not appear to be due to a decrease in the number of Na^+,K^+-ATPase sites, because the amount of phosphoenzyme intermediate formed in the presence of mastoparan was greater than, rather than less than, that formed in its absence. This is supported by the observation that the inhibition occurred rapidly, reaching completion within 30 seconds or less. It therefore does not appear to be due to a decrease in the rate of synthesis of Na^+,K^+-ATPase sites or to an increase in the rate of their degradation since these processes would require a latency of several minutes to hours for their effect to become evident. A possible mechanism for the inhibition of Na^+,K^+-ATPase in the presence of less than saturating concentrations of Na^+, K^+, or ATP, would be a decrease in the enzyme's affinity for one or more of these substrates. However, this does not appear to have occurred in the present experiments, because mastoparan decreased the Kd for K^+, and it had no effect on the Kd's for Na^+ and ATP. Mastoparan also had no effect on the Hill coefficients for the three substrates. Because of the apparent increase in the affinity of the enzyme for K^+ observed in the presence of mastoparan, inhibition of Na^+,K^+-ATPase activity by

mastoparan occurred almost exclusively at the higher range of K^+ concentrations. The effect of the inhibitor on the Kd for K^+ therefore appears to be as important kinetically as its affect on the Vmax in the lower range of K^+ concentrations.

The mechanism by which mastoparan increased the amount of phosphoenzyme intermediate is obscure. A highly speculative explanation for this effect is that even in the presence of 100 mM Na^+ and the absence of added K^+, there was a slow but finite turnover of the phosphoenzyme intermediate, and that mastoparan increased the steady state amount of phosphoenzyme intermediate by slowing its breakdown (the rate limiting step). Stabilization of the phosphoenzyme intermediate could help explain the decrease in Vmax produced by mastoparan since this would decrease the rate of Na^+,K^+-ATPase turnover. Because neomycin, which is an inhibitor of inositol phospholipid metabolism (Lang et al., 1977; Lodhi et al., 1979; Lipsky and Lietman, 1982; Marche et al., 1983; Schacht, 1978; Schwertz et al., 1984), was found to attenuate the inhibition of Na^+,K^+-ATPase by mastoparan, and because the activity of renal Na^+,K^+-ATPase appears to require the presence of phosphatidylinositol (Mandersloot et al., 1978 and Roelofsen and VanLindeSibenius, 1981), the mechanism of the inhibition of Na^+,K^+-ATPase by mastoparan may involve degradation of the phosphatidylinositol "pool".

Chapter 6:

THE EFFECTS OF NEOMYCIN ON MASTOPARAN-INDUCED LACTATE DEHYDROGENASE RELEASE, ETHIDIUM BROMIDE ACCUMULATION, AND INTRACELLULAR FLUORESCEIN DEPLETION IN MDCK CELLS

Introduction

In addition to the many other effects elicited by mastoparan, the peptide also causes the degranulation of mast cells and histamine release (Hirai et al., 1979b) and multigranular exocytosis (Kurihara et al., 1986). One of the possible mechanisms of mastoparan-induced cell injury is the perturbation of membrane phospholipids resulting in an increase in membrane permeability. Presumably the release of lactate dehydrogenase (EC 1.1.1.27; LDH) reflects cell injury and eventual cell death. Although the effect of mastoparan on lactate dehydrogenase release in MDCK cells was examined in previous chapters, no cytological observations using ethidium bromide and fluorescein accumulation were provided to confirm cell death. Accordingly, the effect of mastoparan on LDH release, ethidium bromide, and fluorescein accumulation were studied.

Neomycin, a polycationic aminoglycoside antibiotic, acts on the plasma membrane resulting in the alteration of membrane function (Humes et al., 1982). It interacts with anionic polyphosphoinositides (Sastrasinh et al., 1982). It blocks Ca^{++}-dependent histamine secretion from mast cells (Cockcroft and Gomperts, 1985), inhibits thrombin-induced cell proliferation of hamster fibroblast (Carney et al., 1985), and abolishes thrombin-induced inositol phosphate formation in human platelets (Siess and Lapetina, 1986). The cationicity of the ionizable groups of various aminoglycosides is a major molecular determinant of their

action on plasma membranes (Humes et al., 1982). There is a correlation between the number of cationic charges of the aminoglycoside and its affinity for its membrane receptor. Since mastoparan and neomycin (with 6 ionizable amino groups) are cationic peptides and react with the negatively charged membrane phospholipids, the interaction of mastoparan and other aminoglycosides on membrane permeability was studied.

Results

LDH release in response to different concentrations of mastoparan

LDH release was dependent on the concentration of mastoparan (Figure 6-1a) and the duration of exposure to the toxin (Figure 6-1b). Mastoparan at concentrations of 5 to 75 ug/ml caused marked LDH release. The amount of LDH released peaked at 15 minutes after the addition of mastoparan to the cells.

LDH release from MDCK cells in response to increasing concentrations of neomycin

Neomycin blocks mastoparan-induced LDH release and stabilizes membrane permeability from a dosage of 0.5 mM to a maximum of 2 mM (Figure 6-2a).

Time course of mastoparan-induced LDH release in response to neomycin

Neomycin (2 mM) inhibited LDH release in mastoparan-treated MDCK cells over a 15 minute period (Figures 6-2b). Although neomycin is known to produce nephrotoxic acute renal failure (Humes et al., 1982), a brief exposure of 30 minutes to neomycin alone did not cause LDH release (Figure 6-3). Furthermore, no other cytotoxic effect was observed as demonstrated by ethidium bromide and fluorescein accumulation when MDCK cells were treated with 0.5 to 2.0 mM neomycin (data not shown).

Inhibition of mastoparan-induced LDH release in response to spermidine, streptomycin, gentamicin, and neomycin

The effect of aminoglycosides on mastoparan-induced LDH

release showed that neomycin and gentamicin are more effective than streptomycin (Figure 6-3). In addition, spermidine, a polyamine, inhibits mastoparan-induced LDH release (Figure 6-3).

Effect of mastoparan on percent intracellular fluorescein retention in MDCK cells

Mastoparan (75 ug/ml) depleted intracellular fluorescein; neomycin (2mM) inhibited the action of mastoparan (Figure 6-4). Furthermore, digitoxin (0.5 mM), a cardiac glycoside, did not deplete intracellular fluorescein suggesting that mastoparan and digitoxin act on cell membranes differently (Figure 6-4).

The effect of aminoglycosides on mastoparan-induced ethidium bromide accumulation and intracellular fluorescein depletion correlated with LDH release which showed that neomycin and gentamicin are more effective than streptomycin (Figure 6-4). In addition, spermidine similarly inhibits LDH release and prevents intracellular fluorescein depletion induced by mastoparan (Figure 6-4).

Figure 6-1a. **LDH release in response to different concentrations of mastoparan.** MDCK cells were incubated with mastoparan for 15 minutes at 37°C. Aliquots of the medium were analyzed for LDH (see "Methods"). LDH release was calculated as a percentage of the total cellular LDH (3248 ± 200 units/liter per culture) in unstimulated cultures. Analysis of total cellular LDH was described under "Methods". The values shown in the figure were corrected for spontaneous release to give net release as determined in matched unstimulated cultures. Values shown are mean ± S.E.M. for eight cultures. p values indicated are compared to 0 ug/ml mastoparan; * = $p<.05$; ** $p<.001$.

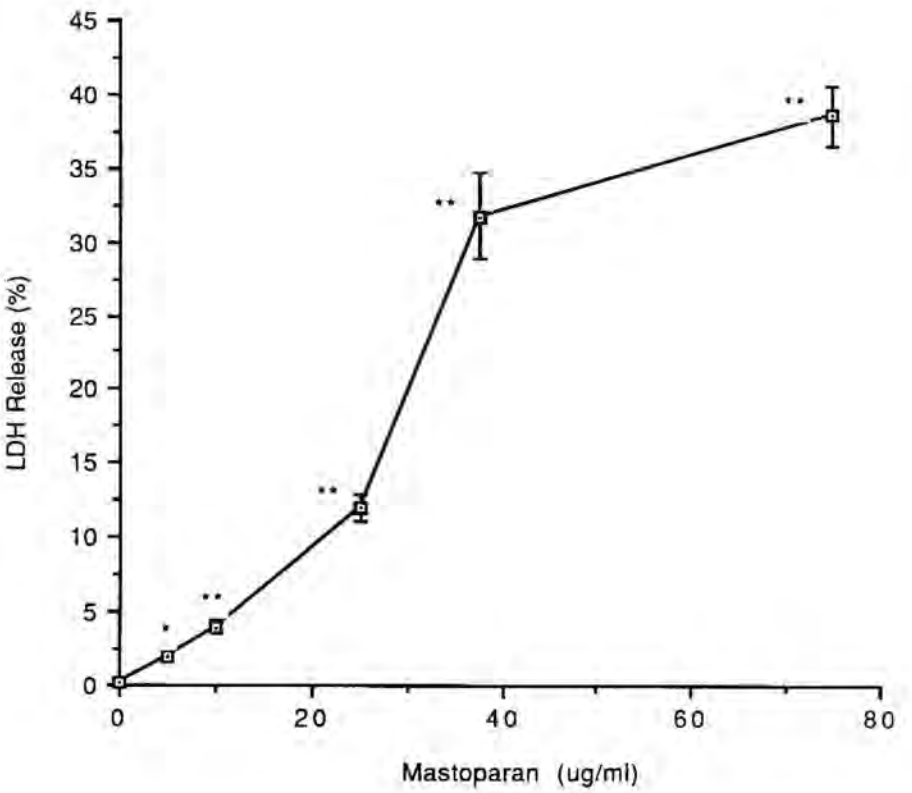

45
40
35
30
25
20
15
10
5
0
LDH Release (%)
0
20
40
60
80
Mastoparan (ug/ml)
*
**
**
**
**

Figure 6-1b. **Time course of LDH release from MDCK cells in response to mastoparan.** MDCK cells were incubated with mastoparan (75 ug/ml, final concentration) at the indicated time at 37°C. Analysis of LDH is described in "Methods". The values are mean ± S.E.M. for eight cultures. p values indicated are compared to time 0. ** = p<.001

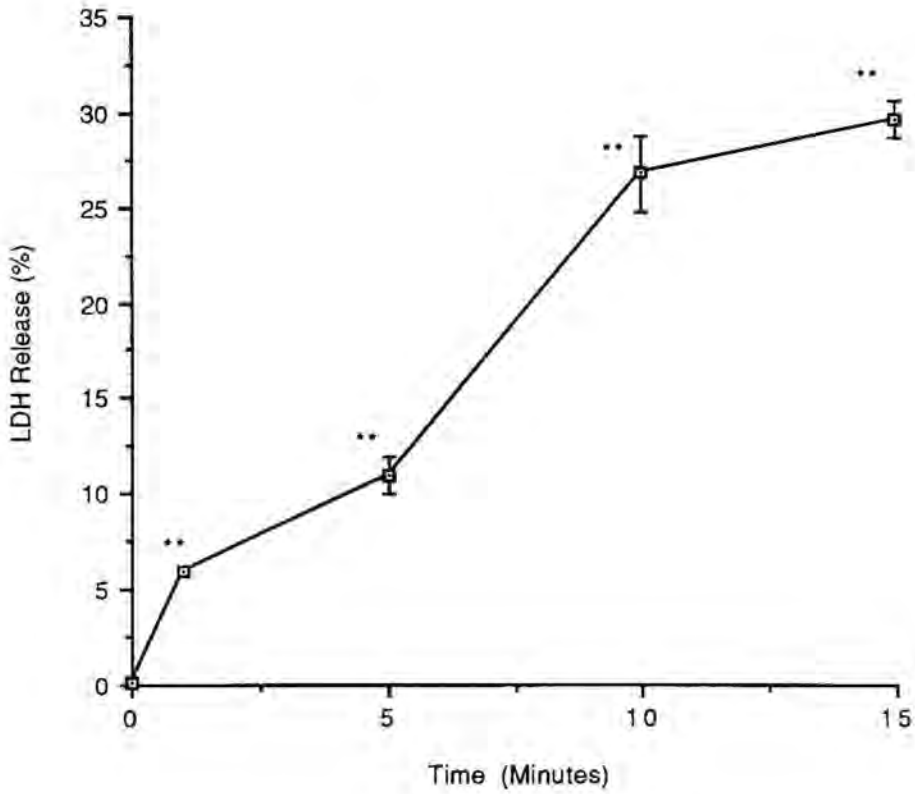

35
30
25
20
15
10
5
0
LDH Release (%)
**
**
**
**
0
5
10
15
Time (Minutes)

Figure 6-2a. **Inhibition of mastoparan-induced LDH release in response to increasing concentrations of neomycin.** MDCK cells were incubated with neomycin for 10 minutes at 37°C followed by the addition of mastoparan (75 ug/ml). After 15 minutes of incubation at 37°C, LDH release was analyzed as described in "Methods". The values are mean ± S.E.M. for eight cultures. p values indicated are compared to neomycin (0 mM); ** = p<.001.

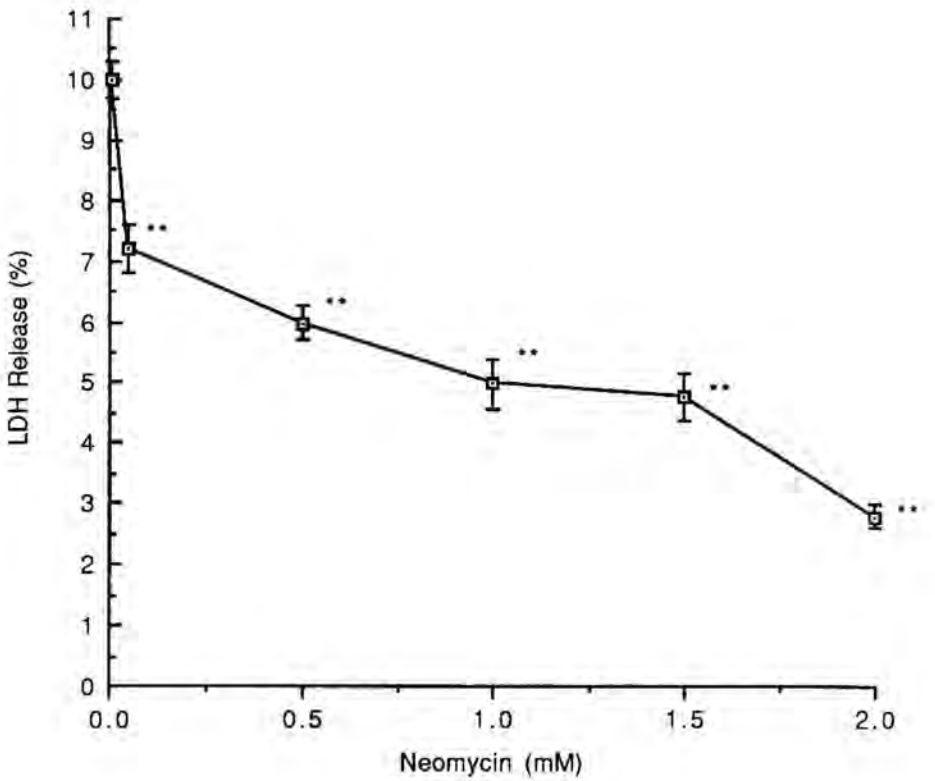

LDH Release (%)
11
10
9
8
7
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
Neomycin (mM)
**
**
**
**
**

Figure 6-2b. **Time course of mastoparan-induced LDH release in response to neomycin.** MDCK cells were incubated with neomycin (2mM; closed squares) or its diluent (open squares) for 10 minutes at 37°C followed by the addition of mastoparan (75 ug/ml). At the indicated time of incubation, LDH release was analyzed as described in "Methods". The values are mean ± S.E.M. Each point (with or without neomycin) represents 8, 12, 4, 8, and 4 cultures at 0.25, 0.50, 5, 10 and 15 minutes, respectively. p values indicated are compared to 2 mM neomycin at each respective time point; ** = $p<.001$.

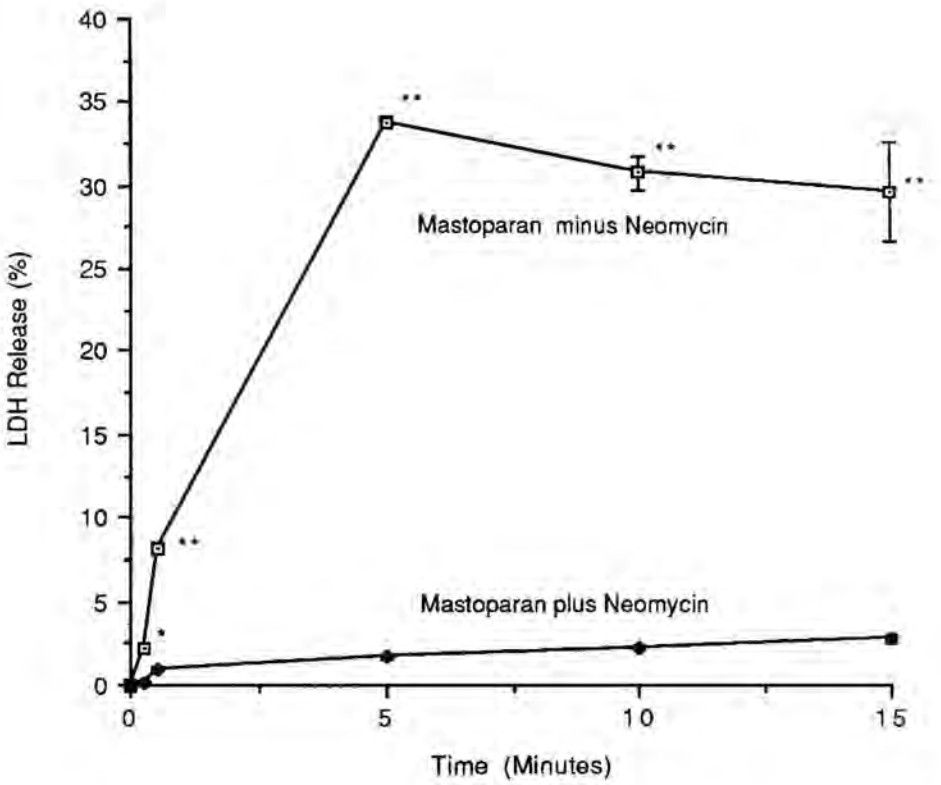

40
35
30
25
20
15
10
5
0
LDH Release (%)
**
**
**
**
*
Mastoparan minus Neomycin
Mastoparan plus Neomycin
0
5
10
15
Time (Minutes)

Figure 6-3. **Inhibition of mastoparan-induced LDH release in response to spermidine, streptomycin, gentamicin, and neomycin.** MDCK cells were incubated with 2 mM spermidine, streptomycin, gentamicin, and neomycin for 10 minutes at 37°C followed by the addition of mastoparan (75 ug/ml) or its diluent. After 15 minutes of incubation, LDH release was analyzed as described in "Methods". The values are mean ± S.E.M. for seven cultures. C = control, M = mastoparan, N = neomycin, G = gentamicin, S = streptomycin, P = spermidine. p values indicated are compared to cells treated with mastoparan and neomycin; N.S. = not significant ($p>.05$); ** = $p<.001$; values representing spermidine treatment alone or antibiotic treatment alone were not significantly different ($p>.05$) from control values.

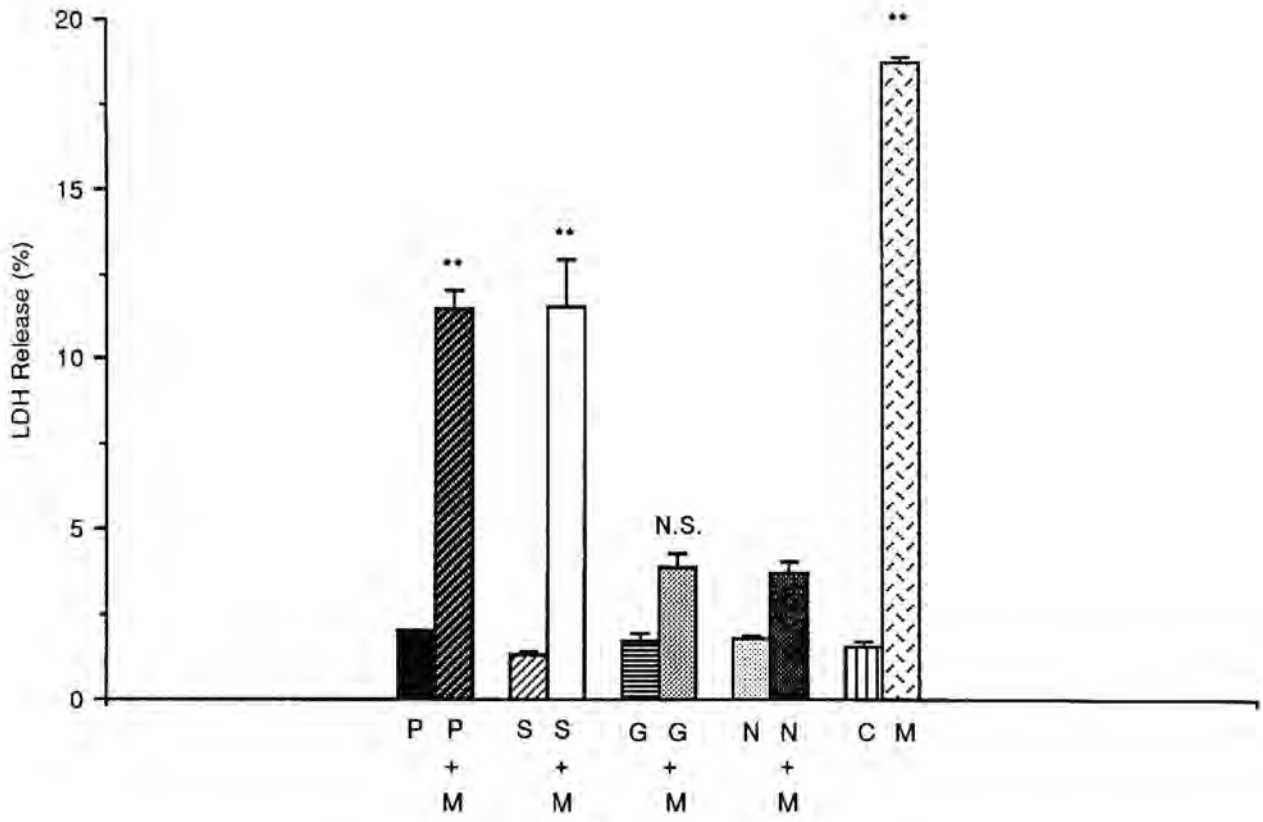
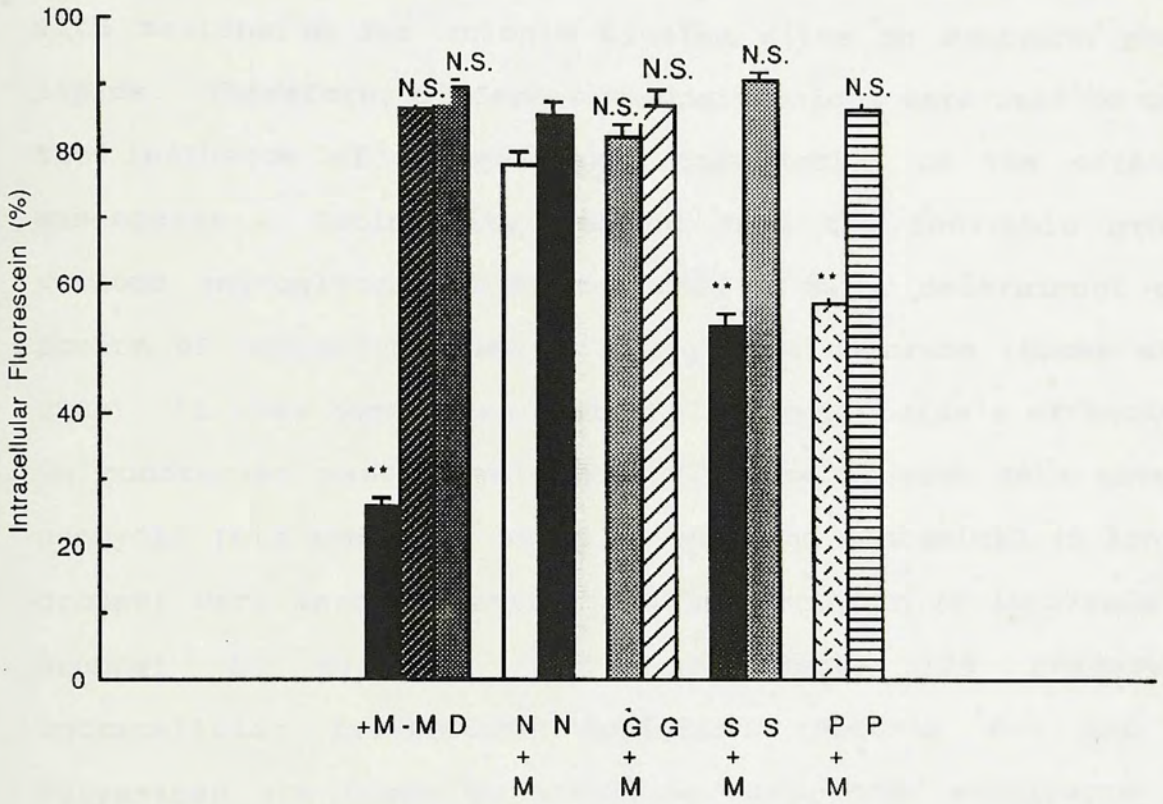

20
15
10
5
0
LDH Release (%)
**
**
**
N.S.
P
P + M
S
S + M
G
G + M
N
N + M
C
M

Figure 6-4. **Effect of mastoparan on percent intracellular fluorescein retention in MDCK cells.** Cell suspension was obtained by treating the mono- layer MDCK cells with trypsin solution for 30 minutes at 37°C as described in "Methods". The cells were incubated with 2 mM neomycin [N], 2 mM gentamicin [G], 2 mM streptomycin [S], 2 mM spermidine [P], and 0.5 mM digitoxin [D] for 5 minutes followed by the addition of mastoparan [M] (75 ug/ml, final concentration) or its diluent. After 15 minutes of incubation, ethidium bromide and fluorescein diacetate were added to the cells and incubated for 3 minutes at room temperature. Cells containing fluorescein (considered as live cells) or ethidium bromide (considered as injured cells) were examined and counted under a fluorescein microscope as described under "Methods". Intracellular fluorescein accumulation was calculated as a percentage of the total number of cells examined. Values representing digitoxin [D], neomycin [N], gentamicin [G], spermidine [P], and streptomycin [S] treatment alone are not significantly different (p.>05) from control values [-M]. The values are mean ± S.E.M. for seven cultures. p values indicated are compared to mastoparan and neomycin treated cells; N.S. = not significant (p>.05); ** = p<.001.


Intracellular Fluorescein (%)
100
80
60
40
20
0
N.S.
N.S.
N.S.
N.S.
N.S.
N.S.
**
**
**
+M -M D
N N
+
M
G G
+
M
S S
+
M
P P
+
M

Discussion

Since both aminoglycosides and mastoparan contain cationic ionizable amino groups, it is possible that neomycin is competing with mastoparan for anionic binding sites on membrane phospholipids. Therefore, different aminoglycosides were used to compare the influence of charge-charge interaction on the effects of mastoparan. Cationicity derived from the ionizable group of various aminoglycosides is not only a major determinant of the action of aminoglycosides on the plasma membrane (Humes et al., 1982), it also correlates with the aminoglycoside's effectiveness in countering mastoparan's effect. The present data show that neomycin (six ionizable amino groups) and gentamicin (5 ionizable groups) were more effective than streptomycin (3 ionizable amino groups) in blocking mastoparan-induced LDH release and intracellular fluorescein depletion (Figures 6-3 and 6-4). Polyamines are known to stabilize membranous structures. Cells treated with the polyamine spermidine (three ionizable amino groups) were also found to inhibit mastoparan-induced LDH release and intracellular fluorescein depletion (Figures 6-3 and 6-4). These results indicate that cationic amino groups play a major role in deterring the actions of mastoparan on membrane permeability.

Chapter 7

Summary and Perspectives

Summary and Perspectives

From the data presented, mastoparan is a cytoactive substance that has the ability to alter various aspects of cell structure and function. Mastoparan has been demonstrated to activate phosphoinositide breakdown by mechanisms that are not yet clear. Though the ionsitol phospholipid precursor of mastoparan-mediated inositol phosphate production and phosphatidylinositol phosphate degradation has been demonstrated in the presence of mastoparan (Figure 3-1a), the activation of phospholipase C by mastoparan has not been established conclusively. In addition, mastoparan is able to activate phosphatidylinositol and phosphatidylinositol 4-phosphate specific kinases in plasma membrane enriched preparations (Tables 3-3, 3-4). This results in the formation of a greater quantity of PIP and PIP_2, respectively, in membrane preparations. However, PIP_2 in the whole (MDCK) cell is degraded; an effect possibly due to intracellular depletion of ATP concentrations.

It is possible that the hydrophobic binding of mastoparan to membrane phospholipids not only effects enzymes involved in phosphoinositide metabolism, but also alters membrane structure and function, and consequently results in an increase in membrane permeability. To verify changes in membrane permeability, cytological observations employing ethidium bromide and fluorescein diacetate were used to corroborate the quantitative data obtained from LDH release. Cell viability was shown to be dramatically lower in the presence of mastoparan when compared to controls

(Figures 6-3, 6-4). The quantity of LDH released was observed to be dose and time dependent (Figure 3-1b, Table 3-1). Furthermore, mastoparan-mediated LDH release and inositol phosphate formation were closely associated to one another and independent of extracellular calcium concentrations (Tables 3-1, 3-2).

Since mastoparan is known to alter membrane structure and function, changes in membrane permeability may not be the only consequences of mastoparan's action. Mastoparan was found to completely inhibit Na^+-dependent net alpha-aminoisobutyric acid (AIB) uptake in MDCK cells (Figures 4-1, 4-2). Since Na^+,K^+-ATPase activity is the energy source for the development and maintenance of the Na^+ gradient, it is possible that mastoparan inhibits AIB uptake by inhibiting Na^+,K^+-ATPase activity.

Mastoparan was found to inhibit rat renal Na^+,K^+-ATPase activity in a time and dose dependent fashion (Figures 5-1, 5-2). At the highest concentration tested (100 ug/ml), the enzyme's Vmax was inhibited by 25%. This inhibition reached completion within 30 seconds. Due to mastoparan's rapid effects on Na^+,K^+-ATPase activity, this inhibition does not appear to involve either a decrease in the rate of synthesis of Na^+,K^+-ATPase sites or an increase in the rate of their degradation since these processes require a latency period of at least several minutes. In addition, the amount of phosphoenzyme intermediate formed in the presence of mastoparan was greater than that formed in its absence (Figure 5-6), further indicating that inhibition of Na^+,K^+-ATPase by mastoparan is not due to a decrease in the number of Na^+,K^+-ATPase sites.

A possible mechanism for the inhibition is that mastoparan stabilizes the phosphoenzyme intermediate and reduces the Vmax of the enzyme by decreasing the rate of turnover of existing enzyme sites.

Though mastoparan has been demonstrated to inhibit Na^+,K^+-ATPase activity, the inhibition of Na^+,K^+-ATPase activity alone does not altogether explain the complete inhibition of net AIB uptake. In the presence of 1 mM ouabain, AIB net uptake was inhibited by 36%, whereas mastoparan (75 ug/ml) completely inhibited net AIB uptake (Figure 4-3). Since 1 mM ouabain is sufficient to completely inhibit Na^+,K^+-ATPase activity, this additional inhibition of AIB net uptake by mastoparan may be due to some mechanism different from, or in addition to, the inhibition of Na^+,K^+-ATPase activity. A possible mechanism is that mastoparan causes an increase in membrane permeability resulting in the dissipation of the Na^+ gradient. This increase in membrane permeability could explain the significant increase in AIB efflux when compared to controls, the release of LDH from the cells, as well as the intracellular accumulation of ethidium bromide in earlier studies.

The mechanisms involved in mastoparan's inhibitory effect on AIB uptake and Na^+,K^+-ATPase activity are still an area of speculation. Mastoparan can either affect cell function directly or its effects could be indirect through the activation of phosphoinositide breakdown. This latter proposal was initially favored based on neomycin's ability to attenuate mastoparan's

inhibitory effects. Aminoglycoside antibiotics are relatively specific inhibitors of inositol phosphate metabolism (Lang et al., 1977; Lodhi et al., 1979; Lipsky and Lietman, 1982; Marche et al., 1983; Schacht, 1978; Schwertz et al., 1984), an effect supported by the obervation of attenuated mastoparan-mediated inositol phosphate formation in the presence of neomycin (Table 3-6). Although the mechanism of neomycin's ability to "protect" cell function was initially thought to be inhibition of inositol phospholipid metabolism, studies employing molecules with different charged groups showed that this is not the case. Cationic groups are a major determinant in deterring the actions of mastoparan on cell function, especially membrane permeability (Figures 6-3, 6-4). Therefore, neomycin's ability to attenuate mastoparan-mediated inhibition of AIB net uptake and Na^+,K^+-ATPase activity and the increase in AIB efflux, LDH release, and intracellular ethidium bromide accumulation, is through charge-charge interactions which prevented the binding of mastoparan to membrane phospholipids.

If mastoparan is permitted to interact with the plasma membrane without interference from polycationic charged groups, it will be able to elicit its cytotoxic effects. However, neomycin's ability to attenuate mastoparan's effects suggests that phosphoinositide breakdown plays a role in the mastoparan-induced alterations in plasma membrane structure and function and the pathogenesis of cell injury. The binding of mastoparan to membrane phospholipids may be required for the stimulation of this breakdown. Ideally a method should be used to inhibit specific enzymes involved in

phosphoinositide breakdown. This would provide the tool necessary for determining the role of phosphoinositide breakdown in mastoparan's pathogenesis. To date, specific inhibitors of enzymes involved in phosphoinositide breakdown that do not compete on a charge-charge basis with mastoparan have not been found.

There are still significant gaps in our knowledge about the regulation of the phosphoinositide pathway as well as the whole biochemical "image" of wasp venoms. Further research on the biochemical mechanism of the toxicity of wasp venoms will therefore be necessary.

Curriculum Vitae

Samuel Peter Eng
[redacted]

Education:
Bachelor of Arts degree from Catholic University in the field of Biology with a cumulative GPA of 3.0 (1983). Master of Science degree in Physiology from the Uniformed Services University of the Health Sciences (USUHS) with a cumulative GPA of 3.3 (Fall 1989). Earned all expenses.

Work Experiences:
During my tenure at the USUHS as a Teaching/Research Assistant, I investigated the role of wasp, bee, and cobra toxins on the phosphoinositide metabolism in Madin-Darby Canine Kidney (MDCK) cells. In addition, I had taught first year level Medical Physiology laboratories which involved surgeries on dogs, pigs, and rats. My primary advisor was Dr. Chu S. Lo (295-3524)
[09/85-Present]
Primary Gain: Augmenting my ability to develop experimental designs in a logical manner. Acquiring surgical skills enabling me to use *in vivo* models in addition to my proficiency in using *in vitro* models.

Prior to entering graduate school, I served as a biological technician at USUHS investigating the role of L-3,5,3' triiodothyronine (T_3) on the Na,K-ATPase activity and amino acid transport in submandibular gland cells. The project was guided by Dr. Chu S. Lo (295-3524).
[9/84-9/85]
Primary Gain: Being able to work independently on research projects. Setting weekly goals and results for presentation.

I also worked at the National Heart, Lung, and Blood Institute. The position studied how specific insect venom peptides (melittin, mastoparan) inhibited calmodulin activated *B. pertussis* adenylate cyclase. Such studies may elucidate how calmodulin activates a varied number of enzymes including *B. pertussis* adenylate cyclase. Advising Post-doctorate was Dr. Vilas Shirdhatti (496-9459).
[1/84-9/84]
Primary Gain: Furthering my experience in enzyme assay systems. Obtaining experience in maintaining biohazardous materials (P1 level), gel electrophoresis, high performance liquid chromatography, Beckman spectrophotometer, and the Perkin-Elmer Flurometer.

During the period spanning the Fall of 1983 to the Spring of 1984, I enrolled in several upper level graduate courses.

I held a teaching assistantship for tuition remittance. These responsibilities included instructional lecture and demonstration regarding immunoelectrophoresis, immunizing animals, plaque assays, and tissue culture. The immediate supervisor was Dr. Ursula Krzych (635-5265). [9/83-5/84]
Primary Gain: Insight into formulating and presenting a concise lecture.

During the Summer of 1983, I returned to the National Cancer Institute to perform ELISAs using methyl umberliferol for flourescent quantitation. Some tissue culture was performed. The position was directed by Dr. Poirier at NCI (496-8898).
Primary Gain: Maintaining techniques used for immunoassay. Gained new perspectives into increasing the sensitivity of such assay systems.

A short term position was held which involved patient work and large time involvements in both field studies and herbarium research. Tasks performed were locating and searching for data pertaining to endangered vascular plants of Virginia and Maryland. This project was under the supervision of Ms. Unzicker, who may be reached at 635-5275. [9/7/81 - 12/15/81]
Primary Gain: Received guidelines necessary for obtaining, extracting, and researching specimens from the "field".

Concommitantly with the position previously stated, I held a volunteer position in the Emergency Room at the Providence Hospital in Washington, D.C. This position required one to be able to obtain vital signs of patients and to be placed in an organized manner to best suit the situation and organization of the emergency room. This position was initiated by Dr. Matta and Sr. Mary Paul who may be reached at 269-7389. [9/1/81 - 1/15/82]
Primary Gain: Became familiarized with critical operation of an emergency room environment. Receiving instruction on obtaining vital signs from patients.

I also held a position at the National Institute of Health, National Cancer Institute, Laboratory of Cellular Carcinogenesis and Tumor Promotion and in vitro Pathogenesis section. I was supervised by Dr. Miriam C. Poirier who may be reached at 496-8898. My primary duty was to assist in the development of sensitive radioimmunassays to detect carcinogens bound to DNA of human subjects exposed to environmental carcinogens. These included performing assays involving antigen-antibody reactions such as the Enzyme-linked Immunosorbent Assay (ELISA). The Radioimmunoassay and the Ultrasensitive Enzymatic Radio-immunoassay (RIA and USERIA, respectively), were also other types of assays with which I have assisted. [9/10/80 - 4/10/82]
Primary Gain: Obtained practical experience in performing enzyme assays. Understanding the basis for using immunoassays to detect environmental carcinogens bound to DNA.

Prior to the position at the National Cancer Institute, I held a position as a research assistant at the Catholic University of America. I was trained and supervised by Dr. Nardone who may be reached at 635-5275. Responsibilities were to assist in the maintenance and study of epithelial cells culture as part of a program in cell toxicity. Tissue culture experience with some training in cloning and chromosome spreads were needed to perform the needed tasks of my position. [10/1/79 - 9/10/80]
Primary Gain: Using in vitro models to study cells exposed to heavy metal exposure (Cd, Pb). Obtained tissue culture qualifications.

Preceding my attendance at Catholic University I served as an assistant manager at Jade Gardens Restaurant supervising 4 employees. My tasks were to assist in the basic directing and conducting the interests or affairs of a business establishment such as Jade Gardens. The manager is Chuck Ng and he may be reached at (301) 760-4428. [Summer 1979]
Primary Gains: Learned how to elicit strong cooperation from others and to schedule work for the employees for best results. Gaining basic management skills.

While attending T.C. Williams High School, I held a volunteer position as a laboratory aide researching in the field of bacterial contamination in streams and heavy metal distribution in plants. Both projects were partially funded by the E.P.A. and were recognized on the state level in Virginia. Other responsibilities included the maintenance of the laboratory and its technical equipment. [9/78-6/79].
Primary Gains: Knowing the skills used in maintaining and operating the Atomic Absorption Spectrophotometer.

Honors:
The 1979 Outstanding student in Advanced Placement Biology, Microbiology, and United States History. Dean's List for Spring Semester 1982. Outstanding Performance as biological technician in 1985. Scholarship to attend graduate school in 1985.

I.) Publications:

A. Abstracts:

1. David Clough, Samuel Eng, Laura Klein, and Chu S. Lo. (1986) "Inhibition of Rat Renal Na,K-ATPase by Anti-Alpha Subunit Polyclonal Antibodies." Fed. Proc. 45(3): 651.

2. C.S. Lo, C. Kim, L.E. Klien, and S. Eng. "Effect of Triiodothyronine and Corticosterone on Primary Cultured Submandibular Gland (Na^+ + K^+)-Adenosine Triphosphatase." Presented at the Poster Session of The 1985 Endocrine Society's annual meeting.

3. Samuel Eng and David Kockler. "The Distribution of Heavy Metals in Plants" The Virginia Junior Academy of Sciences and the Virginia Academy of Science 1979 Meeting at Richmond, Virginia.

B. Manuscripts:

1. Samuel P. Eng, David Clough, and Chu S. Lo. "Inhibition of Alpha-aminoisobutyric acid uptake by mastoparan in Madin Darby Canine Kidney (MDCK) cells." Life Sciences, accepted for publication.

2. Samuel P. Eng, David Clough, and Chu S. Lo. "The Effects of mastoparan on Rat Kidney Na,K-ATPase activity." Life Sciences, submitted for publication.

3. Samuel P. Eng and Chu S. Lo. "Mastoparan increases membrane bound PI kinase and PIP kinase activities in MDCK cells." Life Sciences, accepted for publication.

4. Samuel P. Eng and Chu S. Lo. "Neomycin Inhibits Mastoparan-induced Lactate Dehydrogenase Release, Ethidium Bromide Accumulation, and Intracellular Flourescein Depletion in MDCK cells." Cell Biology and Toxicology, accepted for publication.

5. Howard Bryant, Samuel P. Eng, Laura E. Klein, and Chu S. Lo. (1988) "Effects of triiodothyronine on resting membrane potentials of primary cultured rat submandibular gland cells." Cell Biology International Reports, 12 (12): 1027-1036.

6. Theresa N. Lo, Samuel P. Eng, Leslie A. Jaseph, Michael A. Beaven, and Chu S. Lo. (1988) "Cardiotoxin from cobra venom increases the level of phosphatidyl-inositol 4-monophosphate and phosphatidylinositol kinase activity in two cell lines." Biochimica. et. Biophysica. Acta. 970: 51-60.

7. Samuel P. Eng and Chu S. Lo. (1987) "Effect of triiodothyronine on System A amino acid transport in cells of rat submandibular glands." Pflugers Archiv. 408: 519-523.

8. Vilas Shirhatti, Edward Sokoloski, Samuel Eng, Steven Hench, Franca Riccardi, and Gopal Krishna. (1986) "A Simple Method for the Assay of Bordetella pertussis Adenylate Cyclase Employing ^{31}P Nuclear Magnetic Resonance Spectroscopy." Journal of Cyclic Nucleotide and Protein Phosphorylation Research. 11(2): 137-147.

II.) Manuscripts published which acknowledge my technical assistance:

1. Juichiro Nakayama, Stuart Yuspa, and Miriam Poirier. "Comparison of Benzo(a)pyrene-DNA adduct Formation and Removal in Mouse Epidermis in vivo and Mouse Keratinocytes in vitro, and the relationship of DNA binding to the Initiation of Skin Carcinogenesis." In press.

2. Frederica Perera, Miriam Poirier, Stuart Yuspa. 1982 "A pilot project in molecular epidemiology: determination of benzo(a)pyrene-DNA adducts in animal and human tissues by immunoassays." Carcinogensis 3(12): 1405-1410., 1982.

3. Miriam Poirier, John Hunt, B'Ann True, and Brian Laisher. "Kinetics of DNA Adduct Formation and Removal in Liver and Kidney of Rats Fed 2-Acetylaminoflourene" in Extrahepatic Drug Metabolism and Chemical Carcinogenesis, J. Rdystrom, J. Montelius, and M. Bengtsson eds.

REFERENCES

Argiolas, A. and J.J. Pisano. 1983. Facilitation of phospholipase A_2 activity by mastoparans: A new class of mast cell degranulating peptides from wasp venom. J. Biol. Chem. 258: 13697-13702.

Arnold, R.E. 1973. Hymenoptera-Flying Insects. In: What To Do About Bites and Stings of Venomous Animals, Macmillan Publishing Co., New York. pp. 5-15.

Banks, B.E.C., J.M. Hanson, and N.M. Sinclair. 1976. The isolation and identification of noradrenaline and dopamine from the venom of the honey bee, Apis mellifica. Toxicon 14: 117-125.

Barr, S.E. 1974. Allergy to hymenoptera stings. JAMA 228(6): 718-720.

Beaven, M., J. Moore, G. Smith, T. Hesketh, and J Metcalfe. 1984. The calcium signal and phosphatidylinositol breakdown in 2H3 cells. J. Biol. Chem. 259(11): 7137-7142.

Berridge, M.J. 1983. Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyze polyphosphoinositides instead of phosphatidylinositol. Biochem. J. 212: 849-858.

Berridge, M.J., R.M.C. Dawson, C.P. Downes, J.R. Heslop, and R.F. Irvine. 1983. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem. J. 212(2): 473-482.

Bhoola, K.D., J.D. Calle, and M. Schachter. 1961. Identification of acetylcholine, 5-hydroxytryptamine, histamine and a new kinin in hornet venom (Vespa crabro). J. Physiol. (Lond.) 159: 167-182.

Buckley, J.T. and J.N. Hawthorne. 1972. Erythrocyte membrane polyphosphoinositide metabolism and the regulation of calcium binding. J. Biol. Chem. 247: 7218-7223.

Burgess, G.M., P.P. Godfrey, J.S. McKenney, M.J. Berridge, R.F. Irvine, and J.W. Putney. 1984. The second messenger linking receptor activation to internal Ca^{++} release in liver. Nature 309: 63-66.

Camoratto, A.M. and L. Grandison. 1985. Evidence supporting a correlation between arachidonic acid release and prolactin secretion from GH3 cells. Endocrinology 116(4): 1506-1513.

Carney, D.H., D.L. Scott, E.A. Gordon, and E.F. LaBelle. 1985. Phosphoinositides in mitogenesis: Neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. Cell 42: 479-488.

Christensen H.N. and M.E. Handlogten. 1977. Na^{+}/Li^{+} selectivity in transport A: effects of substrate structure. J. Membr. Biol. 37(3-4): 193-211.

Christensen, H.N. 1975. Biological transport In: Benjamin, W.A. (ed). Addison-Wesley, Reading, Massachusetts, USA.

Cockcroft, S., J.A. Taylor, and J.D. Judah. 1985. Subcellular localization of inositol lipid kinase in rat liver. Biochim. Biophys. Acta. 845: 163-170.

Cockcroft, S. and B.D. Gomperts. 1985. Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature 314(6011): 534-536.

Downes, C.P. and R.H. Michell. 1981. The polyphosphoinositide phosphodiesterase of erythrocyte membranes. Biochem. J. 198(1): 133-140.

Edery, H., J. Ishay, I. Lass, and S. Gitter. 1972. Pharmacological activity of oriental hornet (Vespa orientalis) venom. Toxicon 10: 13-23.

Edery, H., J. Ishay, S. Gitter, and H. Joshua. 1978. Venoms of Vespidae. In: Arthropod Venoms, S. Bettini (Ed.). Springer-Verlay, Berlin. pp. 691-771.

Edidin, M. 1970. A rapid, quantitative flourescence assay for cell damage by cytotoxic antibiotics. J. Immunol. 104(5): 1303-1306.

Eng, S.P. and C.S. Lo. 1987. Effect of triiodothyronine on System A amino acid transport in cells of rat submandibular glands. Pflugers Archiv. 408: 519-523.

Fiske; C. and Y. Subbarow. 1925. The colormetric determination of phosphorous. J. Biol. Chem. 66: 375-400.

Garrett, R.J.B. and C.M. Redman. 1975. Localization of enzymes involved in polyphosphoinositide metabolism on the cytoplasmic surface of the human erythrocyte membrane. Biochim. Biophys. Acta. 382: 58-64.

Geller, R.G., H. Yoshida, M.A. Beavan, Z. Horakova, F.L. Atkins, H. Yamabe, and J.J. Pisano. 1976. Pharmacologically active substances in the venoms of the bald-faced hornet, Vespula(Dolichovespula) maculata, and the yellow jacket, Vespula (Vespula) maculifrons. Toxicon. 14(1): 27-33.

Golden, D.B., D.G. Marsh, A. Kagey-Sobotka, L. Freidhoff, M. Szklo, M.D. Valentine, and L.M. Lichtenstein. 1989. Epidemiology of insect venom sensitivity. JAMA 262: 240-244.

Habermann, E. 1972. Bee and wasp venom: The biochemistry and pharmacology of their peptides and enzymes are reviewed. Science. 177: 314-322.

Habermann, E. and W.P. Neumann. 1957. Reinigung der Phospholipase A des Bienengiftes. Biochem. Z. 328: 465-473.

Higashijima, T., S. Uzu, T. Nakajima, and E.M. Ross. 1988. Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP binding regulatory proteins (G proteins). J. Biol. Chem. 263(14): 6491-6494.

Higashijima, T., K. Wakamatsu, M. Takemitsu, M. Fujino, T. Nakajima, and T. Miyazawa. 1983. Conformational change of mastoparan from wasp venom on binding with phospholipid membrane. FEBS-Lett 152(2): 227-230.

Hirai, Y., T. Yasuhara, H. Yoshida, and T. Nakajima. 1981. A new mast-cell degranulating peptide, mastoparan-M, in the venom of the hornet vespa-mandarinia. Biomed. Res. 2 (4): 447-449.

Hirai, Y., Y. Ueno, T. Yasuhara, H. Yoshida, and T. Nakajima. 1980. Mast-cell degranulating peptide, polistes mastoparan, in the venom of polistes-jadwigae. Biomed. Res. 1 (2): 185-187.

Hirai, Y., M. Kuwada, T. Yasuhara, H. Yoshida, and T. Nakajima. 1979a. A new mast cell degranulating peptide homologous to mastoparan in the venom of Japanese hornet (Vespa xanthoptera). Chem. Pharm. Bull. (Tokyo) 27: 1945-1946.

Hirai, Y., T. Yasuhara, H. Yoshida, T. Nakajima, N. Fujino, and C. Kitada. 1979b. A new mast degranulating peptide "mastoparan" in the venom of Vespula lewisii. Chem. Pharm. Bull. (Tokyo), 27: 1942-1944.

Hokin, L.E. 1985. Receptors and phosphoinositide generated second messengers. Ann. Rev. Biochem. 54: 205-235.

Humes, H.D., J.M. Weinberg, and T.C. Knauss. 1982. Clinical and pathophysiologic aspects of aminoglycoside nephrotoxicity. Am. J. Kidney Dis. 2(1): 5-29. (Review).

Ishay, J.S. 1978. Anticholinesterase-like activity by oriental hornet (Vespa orientalis) venom and venom sac extract. Experientia 35: 636-639.

Ishay, J.S., Z. Abraham, Y. Grunfield, and S. Gitter. 1974. Catecholamines in social wasps. Comp. Biochem. 48(2A): 369-373.

Jacques, R. and M. Schachter. 1954. The presence of histamine, 5-hydroxytryptamine and a potent slow contracting substance in wasp venom. Br. J. Pharmacol. 9: 53-57.

Jolles J., H. Zweirs, A. Dekker, K. Wirtz, and W. Gispen. 1981. Corticotropin-(1,24)-teracosapeptide affects protein phosphorylation and polyphosphoinositide metabolism in rat brain. Biochem. J. 194(1): 283-291.

Jorgensen, P.L. 1974. Purification and characterization of Na^+,K^+-ATPase. III. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphate. Biochim. Biophys. Acta. 356: 36-52.

King, T.P., A.K. Sobotka, A. Alagon, L. Kochoumian, and L.M. Lichtenstein. 1978. Protein allergens of white-faced hornet, yellow hornet, and yellow jacket venoms. Biochem. 17: 5165-5174.

King, T.P., A.C. Alagon, J. Kuan, A.K. Sobotka, and L.M. Lichtenstein. 1983. Immunochemical studies of yellow jacket venom proteins. Molec. Immunol. Abstract 20: 297.

Kishimura, H., T. Yasuhara, H. Yoshida, and T. Nakajima. 1976. Vespakinin-M a novel bradykinin analogue containing hydroxyproline, in the venom of Vespa mandarinia Simith. Chem. Pharm. Bull. 24: 3896-3897.

Knoppél, E., D. Eisenberg, and W. Wickner. 1979. Interaction of melittin, a preprotein model, with detergents. Biochemistry 18: 4177-4181.

Kolesnick, R.N., I. Musacchio, C. Thaw, and M.C. Gershengorn. 1984. Arachidonic acid mobilizes calcium and stimulates prolactin secretion from GH3 cells. Am. J. Physiol. 246(5): E458-462.

Kolesnick, R.N., I. Musacchio, C. Thaw, and M.C. Gershengorn. 1984. Thyrotropin (TSH)-releasing hormone decreases phosphatidylinositol and increases unesterified arachidonic acid in thyrotropic cells: possible early events in stimulation of TSH secretion. Endocrinology 114(2): 671-676.

Kurihara, H., K. Kitajima, T. Senda, H. Fujita, and T. Nakajima. 1986. Multigranular exocytosis induced by phospholipase A2-activators, melittin and mastoparan, in rat pituitary cells. Cell. Tissue Res. 243(2): 311-316.

Kuroda, Y., M. Yoshioka, K, Kumakura, K. Kobayashi, and T. Nakajima. 1980. Effects of peptides on the release of catecholamines and adenine nucleotides from cultured adrenal chromaffin cells. Proc. Jpn. Acad. 56(B): 660-664.

Lad, P.L. and W.T. Shier. 1980. Effect of melittin-induced membrane alterations on rat heart adenylate cyclase activity. Arch. Biochem. Biophys. 204: 418-424.

Lang, V., G. Pryhitka, and J. Buckley. 1977. Effect of neomycin and ionophore A23187 on ATP levels and turnover of polyphosphoinositides in human erythrocytes. Can. J. Biochem. 55: 1007-1012.

Lipsky, J.J. and P.S. Lietman. 1982. Aminoglycoside inhibition of renal phosphatidylinositol phospholipase C. J. Pharmacol. Exp. Ther. 220: 287-292.

Lo C.S., T.R. August, U.A. Liberman, and I.S. Edelman. 1976. Dependence of renal Na^+,K^+-adenosine triphosphatase activity on thyroid status. J. Biol. Chem. 251: 7826-7833.

Lodhi, J., N.D. Weiner, and J. Schacht. 1979. Interaction of neomycin with monomolecular films of polyphosphoinositides and other lipids. Biochim. Biophys. Acta. 557: 1-8.

Lowry, O.H., N.J. Roebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurment with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Maeyama, K., J. Cunhamel, R. Hohman, H. Metzger, and M. Beaven. 1986. Hydrolysis of inositol phospholipids as an indicator of IgE receptor mediated events in rat basophil (2H3) cell cultures. Fed. Proc. 45(3): 243.

Mandersloot, J.G., B. Roelofsen, and J. DeGier. 1978. Phosphatidylinositol as the endogenous activator of the (Na^+,K^+)-ATPase in microsomes of rabbit kidney. Biochem. Biophys. Acta. 508(3): 478-485.

Marche, P., S. Koutouzov, and A. Girard. 1983. Impairment of membrane phosphoinositide metabolism by aminoglycoside antibiotics: streptomycin, amikacin, kanamycin, dibekacin, gentamicin, and neomycin. J. Pharmacol. Exp. Ther. 227: 415-420.

Maschwitz, U., and M. Muhlenbe. 1975. Strategy of predation in some oriental Leptogemy species (Formicidae-Ponerinae). Oecologia. 20(1): 65-83.

Michell, R.H. 1975. Inositol phospholipids and cell-surface receptor function. Biochim. Biophys. Acta. 415(1): 81-147.

Michell, R.H., J. Harwood, R. Coleman, and J. Hawthorn. 1967. Characteristics of rat liver phosphatidylinositol kinase and its presence in plasma membrane. Biochim. Biophys. Acta. 144: 649-667.

Michener, C. and D. Brothers. 1974. Were workers of eusocial hymenoptera initially altruistic or oppressed? P. Nas. Us. 71(3): 671-674.

Miyazawa, T. and J. Higashij. 1984. Conformational change of mastoparan from wasp venom on binding with phospholipid membranes. Biomed. Res. 5(5): 169-174.

Mollay, C., G. Kreil, and H. Berger. 1976. Action of phospholipase A2 on the cytoplasmic membrane of E. coli: Stimulation by melittin. Biochim. Biophys. Acta. 426: 317-324.

Nakajima, T., T. Yasuhara, N. Yoshida, Y. Takemoto, S. Shinonaga, R. Kano, and H. Yoshida. 1983. The pattern analysis of biologically active amines in some hymenopteran venoms by high performance liquid chromatography. Jpn. J. Sanit. Zool. 34: 61-71.

Nakajima, T. 1984. Biochemistry of vespid venom. In: Handbook of Natural Toxins: Insect poisons, allergens, and other invertebrate venoms, Vol. II., Tu, A. (ed), Marcel Dekker, Inc. New York. pp. 109-134.

Naor, Z. and K.J. Catt. 1981. Mechanism of action of gonadotropin-releasing hormone. Involvement of phospholipid turnover in luteinizing hormone release. J. Biol. Chem. 256(5): 2226-2229.

Nazimov. I.V., L.G. Snezhkova, and A.T. Miroshnikov. 1980. Structure and properties of mastoparan. II. An oligopeptide from the venom of Vespa orientalis hornet. Proc. 3d Symp. Chem. Pept. Prot. USSR FRG. pp. 19-25.

O'Connor, R. and L. Peck. 1980. Bee sting: The chemistry of an insect venom. J. Chem. Educ. 57: 206-209.

Okano, Y., H. Takagi, T. Tohmatsu, S. Nakaskima, Y. Kuroda, K. Saito, and Y. Nozawa. 1985. A wasp venom mastoparan induced polyphosphoinositide breakdown in rat peritoneal mast cells. FEBS-Lett. 188(2): 363-366.

Okumura, K., K. Inui, Y. Hirai, and T. Nakajima. 1981. The effect of mastoparan on ion movement in black lipid membrane. Biomed. Res. 2: 450-452.

Olson, F.C., D. Munjal, and A.N. Malviya. 1974. Structural and respiratory effects of melittin (apis mellifera) on rat liver mitochondria. Toxicon 12: 419-425.

Owen, M.D. 1971. Insect venoms: Identification of dopamine and noradrenaline in wasp and bee stings. Experientia 27: 544-545.

Owen, M.D. and A.R. Bridges. 1982. Catechclamines in honey bee (Apis mellifera) and various vespid (hymenoptera) venoms. Toxicon 20: 1075-1084.

Reinert, M. 1936. Zur Kenutnis des Bienengifies Festschrift. Emil Barell, Basel. pp. 1-9.

Rocha e Silva, M., W.T. Beraldo, and G. Rosenfeld. 1949. Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. Am. J. Physiol. 156: 261-273.

Roelofsen, B. and T.M. VanLindeSibenius. 1981. The fraction of phosphatidylinositol that activates the (Na^+,K^+-ATPase in rabbit kidney microsomes is clearly associated with the enzyme protein. Biochim. Biophys. Acta. 647(2): 302-306.

Rotman, B. and B.W. Papermaster. 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of flourogenic esters. Proc. Natl. Acad. Sci. 55(1): 134-141.

Sastrasinh, M., T.C. Knauss, J.M. Weinberg, and H.D. Humes. 1982. Identification of aminoglycoside binding site in rat renal brush border membranes. J. Pharmacol. Exp. Ther. 222(2): 350-358.

Schacht, J. 1978. Purification of polyphosphoinositides by chromatography on immobilized neomycin. J. Lipid Res. 19: 1063-1067.

Schachter, M. 1970. Discovery of non-mammalian kinins. In: Bradykinin, Kallidin and Kallikrein. Handbook of Experimental Pharmacology, Vol. XXV, E.G. Erdos (Ed.). Springer-Veriag, Berlin. 585-588.

Schachter, M. and E.M. Thain. 1954. Chemical and pharmacological properties of the potent, slow contracting substance (kinin) in wasp venom. Br. J. Pharmacol. 9: 352-359.

Schanne, F.A.X., A.B. Kane, E.E. Young, and J.L. Farber. 1979. Calcium dependence of toxic cell death: A final common pathway. Science. 206: 700-702.

Schrey, M.P. and R.P. Rubin. 1979. Characterization of a calcium-mediated activation of arachidonic acid turnover in adrenal phosopholipids by corticotropin. J. Biol. Chem. 256(21): 11234-11241.

Schwartz A., J.C. Allen, and S. Harigaya. 1969. Possible involvement of cardiac Na^+, K^+-adenosine triphosphatase in the mechanism of action of cardiac glycosides. J. Pharmacol. Exp. Ther. 168(1): 31-41.

Schwertz D.W., J.I. Kreisberg, and M.A. Venkatachalam. 1984. Effects of aminoglycosides on proximal tubule brush border membrane phosphatidylinositol-specific phospholipase C. J. Pharmacol. Exp. Ther. 231(1): 48-55.

Sessa, G., J.H. Freer, G. Colacicco, and G. Weissman. 1969. Interaction of a lytic polypeptide, melittin, with lipid membrane systems. J. Biol. Chem. 244: 3575-3582.

Shein, W.T. 1982. Cytolytic mechanisms: Self-destruction of mammalian cells by activation of endogenous hydrolytic enzymes. 1982. J. Toxicol. Toxin Review. 1: 1-32.

Shier, W.T. 1979. Activation of high levels of endogenous phospholipase A2 in cultured cells. Proc. Natl. Acad. Sci. USA 76: 195-199.

Shier, W.T. 1985. The final steps to toxic cell death. J. Toxicol. Toxin Review. 4(2): 191-249.

Shipolini, R.A., G.L. Callewaert, R.S. Cottrell, S. Doonan, C.A. Vernon, and B.E.C. Banks. 1971. Phospholipase A_2 from bee venom. Eur. J. Biochem. 20(4): 459-468.

Siess, W., and E.G. Lapetina. 1986. Neomycin inhibits inositol phosphate formation in humnan platelets stimulated by thrombin but not other agonists. FEBS-Lett. 207: 53-57.

Slivka, S.R. and P.A. Insel. 1987. Alpha-adrenergic receptor mediated phosphoinositide hydrolysis and prostaglandin E_2 formation on Madin-Darby Canine Kidney Cells. J. Biol. Chem. 262: 4200-4207.

Smith, M.T., H. Thor, and S. Orrenius. 1981. Toxic injury to isolated hepatocytes is not dependent on extracellular calcium. Science 213: 1245-1259.

Snedecor, G.W. and G.W. Cochran. 1980. Statistical Methods 6th edition. Ames, Iowa, Iowa State University Press. pp. 26-37; 64-79; 83-102; 194-210.

Sullivan, T.J. 1986. Treatment of reactions to insect stings and bites. In: Levine, M., Lockey, R. (eds). Monograph on Insect Allergy, Dave Lambert Associates, Pittsburgh, PA. pp. 45-53.

Terwilliger, T.C. and D. Eisenberg. 1982a. The structure of melittin. I. Structure determination and partial refinement. J. Biol. Chem. 257: 6010-6014.

Terwillliger, T.C. and D. Eisenberg. 1982b. The structure of melittin. II. Interpretation of the structure. J. Biol. Chem. 257: 6015-6022.

Tu, A.T. 1977. Venom of bees, hornets and wasps. In: Venoms: chemistry and molecular biology. New York, John Wiley and Sons. pp. 502-534.

Udenfriend, S., T. Nakajima, J.J. Pisano. 1967. Structure of the major kinin in wasp (Polistes) venom. Proc. 7th Int. Cong. Biochem. Abstracts VIII(4): 501 (Tokyo).

Ueno, Y, Y. Hirai, T. Yasuhara, H. Yoshida, and T. Nakajima. 1977. On the kinin-like substances in the venom of paper wasp. Proc. 97th Ann. Meeting Pharmaceutical Soc. Japan (Tokyo). v3. p. 37.

Watanabe, M., T. Yasuhara, T. Nakajima. 1976. Occurrence of Thr6-bradykinin and its analogous peptide in the venom of Polistes rhothneyiiwatai V. der Vecht. In: Animal, Plant and Microbial Toxins, Vol II, A. Ohsaka, K. Hayashi, and Y. Sawai(Eds.). Plenum, New York. pp. 105-112.

Yasuhara, T., H. Yoshida, and T. Nakajima. 1977. Chemical investigation of the hornet (Vespa xanthoptera Cameron) venom. The structure of a new bradykinin analogous "Vespakinin-X". Chem. Pharm. Bull. 25: 935-941. Pierce Chemical Company, Rockford, Illinois.

Yasuhara, T., T. Nakajima, and V. Erspamer. 1983. Isolation and sequence analysis of peptides in the picomolar level. In: Peptide Chemistry 1982, S. Sakakibara(Ed.). Protein Res. Foundation, Osaka. 213-218.

Yoshida, H., R.G. Geller, and J.J. Pisano. 1976. Vespulakinis: New carbohydrate-containing bradykinin derivatives. Biochemistry 15(1): 61-64.

Yunes, R., A.R. Goldhammer, W.K. Garner, and E.H. Cordes. 1977. Phospholipases: Melittin facilitation of bee venom phospholipase A2 catalyzed hydrolysis of unsonicated liposome. Arch. Biochem. Biophys. 183: 105-112.